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LEUCOCYTE GROUPING

A METHOD AND ITS APPLICATION

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LEUCOCYTE GROUPING

A method and its application

DRUKKERIJ PASMANS - v. d. VENNESTRAAT 76 - DEN HAAG

This study was carried out in the Blood Bank of the University Hospital, and the Department of Immuno-haematology, Clinic of Internal Medicine, University of Leyden, Netherlands.

LEUCOCYTE GROUPING

A METHOD AND ITS APPLICATION

PROEFSCHRIFT

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Chapter one

GENERAL INTRODUCTION

Antibodies against leucocytes can be formed after multiple blood transfusions and pregnancies. That blood transfusions are a causative factor in the formation of these antibodies has been known since 1954 (2, 11, 19, 34, 43, 44, 54). In 1958 it was realised that formation of antibodies against leucocytes could also be stimulated by pregnancy (55, 58, 59). This conclusion was reached as the result of two observations.

The first observation concerned a transfusion reaction (58): on April 11th 1958 Mrs. v.d.H.B. (age 26 years, VK 246/1958, Department of Obstetrics, University Hospital, Leyden) was delivered of premature twins. Shortly after delivery a post partum haemorrhage necessitated an emergency blood transfusion. Within one hour of the start of the transfusion a severe transfusion reaction manifested itself by collapse, hypotension, nausea, vomiting and a shaking chill.

Investigation of the reaction did not show the presence of blood-group incompatibility. On the other hand the presence of strong agglutinins, which could agglutinate the leucocytes of the donor, were demonstrated.

It is known that leucocyte agglutinins in the blood of a patient who is transfused can cause a transfusion reaction (9, 22, 44, 65). It was therefore concluded that these agglutinins precipitated the transfusion reaction.

On inquiry it appeared that the patient had not been transfused before. The possibility was therefore considered that these agglutinins were formed during the six previous pregnancies (58). This hypothesis proved to be correct (59, 60).

The second observation was made by Payne and Rolfs, who independently and almost simultaneously came to a similar conclusion (55). They found evidence of the importance of pregnancy in the formation of leucocyte agglutinins during a study on the role of blood transfusion in leucocyte agglutinin formation.

Up to the present ten papers have appeared on this subject (25, 31, 55, 56, 57, 58, 59, 60, 63, 64). It was demonstrated that these agglutinins are iso-immune antibodies with group-specific properties. Their mode of formation is in many ways reminiscent of that of the Rhesus antibodies. It could also be shown that leucocyte agglutinins formed during pregnancy can be the cause of non-haemolytic transfusion reactions.

The novel aspect of these findings was the observation concerning the role of pregnancy in the formation of leucocyte agglutinins; these studies also provided a confirmation and an extension of the original observations made by Dausset (19), Killmann (34), van Loghem et al. (44), Spielmann (71) and the American workers Brittingham and Chaplin (9) and Payne (54). It lies outside the scope of this study to cover the literature in extenso. Walford gives a complete survey with 567 references up to the middle of 1959 (74).

In several publications on leucocyte agglutinins formed as a result of pregnancy it was pointed out that these agglutinins are to be pre-

ferred to the agglutinins formed after multiple blood transfusions for the definition of leucocyte groups, as the former arise in response to a known and constant source of antigen (55, 59).

The existence of leucocyte groups has been demonstrated by four separate sets of observations. In the first place leucocyte agglutinins formed after blood transfusions agglutinated leucocytes from a varying number of random donors, but did not agglutinate the leucocytes of the patient who had formed the agglutinin (19). Dausset, studying the agglutination pattern of a group of 27 sera with a panel consisting of 20 leucocyte samples, noticed that 7 of the 27 sera showed more or less the same agglutination pattern (22). From the observation that the leucocytes from these patients were not agglutinated by their own serum nor by the 6 other sera from this group, Dausset concluded that they lacked the same antigen, which he designated Mac. Further proof of the existence of the antigen Mac was furnished by the agglutination pattern of leucocyte agglutinins formed after repeated injections of blood from three donors into six patients. The antigen Mac could be demonstrated in about 60% of the leucocyte samples from randomly selected donors. Dausset's conclusion was that although the existence of a leucocyte group was certain, the anti-Mac sera always contained a mixture of agglutinins of different specificity. Only 2 of the 7 sera showed identical agglutination patterns, i.e. the other sera also agglutinated leucocyte samples which ought not to be agglutinated. It is not known if this was because the sera contained a mixture of antibodies or because the agglutinin anti-Mac failed to agglutinate all Mac-positive leucocytes, as no cross-absorption experiments were done. Reliable grouping of leucocytes was still not possible. Probably for that reason family studies with anti-Mac were not performed either.

In the same paper Dausset reports a second observation which provides additional evidence that leucocyte groups do exist. Monozygotic twins proved to have identical agglutination patterns of their leucocytes, while the agglutination pattern of the leucocytes of dizygotic twins was not identical. Lalezari reported similar findings (40).

A third argument that leucocyte groups do exist was found in family studies, which showed that leucocyte iso-antigens could be inherited (55, 57, 60, 64).

Finally the formation of leucocyte agglutinins during pregnancy, as mentioned above, was a strong argument in favour of the existence of leucocyte groups (55, 60).

From the results of cross-absorption experiments with three sera with leucocyte agglutinins formed during pregnancy van Rood et al. postulated the existence of three other leucocyte groups, leucocyte groups Two, Three and Four (59, 60, 63).

Although the existence of leucocyte groups is now generally accepted, so far no leucocyte group has been satisfactorily defined, neither has a method been described by which this could be achieved.

It is the aim of this thesis to describe how a leucocyte group can be defined. As this is mainly a problem of technique the properties of the two leucocyte agglutination tests in general use (i.e. the one using leucocytes obtained from defibrinated blood (20) and the other using leucocytes obtained from blood made incoagulable with EDTA(44)) will be discussed first.

Chapter two

MATERIALS AND METHODS

Glassware and reagents

All glassware employed in this study was prepared from normal soda-glass. The following items were used.

Centrifuge tubes, 90 x 14 mm.
Round bottomed tubes, 50 x 7 mm, so-called "rhesus tubes".
Round bottomed tubes, 150 x 14 mm (type C).
Conical flasks, 50 ml.
Glass beads, diameter 4 mm.
Microscope slides, 25 x 75 mm and 50 x 300 mm.
Capillary pipettes.

Glassware was cleaned as follows. Immediately after use it was placed in a pail filled with tap-water in order to prevent the drying up of the blood or serum. At the end of the day most of the blood or serum still adhering was removed by rinsing under running tap-water. During the night the glassware was soaked in a 2.5% solution of the detergent Bosmanite AL-25 (Rogier Bosman, Rotterdam) in tap-water. If necessary the glassware was cleaned with a brush next morning. After another rinse in tap-water, the glassware was placed in distilled water for 15 minutes. Thereafter the glassware was dried in an oven at 250°C for 60 minutes. The prevention of the drying up of the blood or serum proved to be essential.

Glassware was siliconised as follows. It was filled with a solution of silicone oil 200/350 centistokes (Dow - Corning Corp., Midland, Mich., U.S.A.) 2% v/v in carbon tetrachloride and the silicone solution was immediately transferred to another tube or container. Glassware that had been in contact with the silicone was dried at 250°C for 60 minutes.

The following solutions were used.

A sterile, but not pyrogen-free solution of 0.15 M NaCl in distilled water buffered with 0.0013 M Na_2HPO_4-NaH_2PO_4 to pH 7.2. This solution will be referred to as saline.
Disodium ethylenediaminotetraacetate, 5% in unbuffered saline; for this solution the abbreviation EDTA will be used.
Sodium azide, 10% solution in saline.
Tween 80, 2% solution in saline.
6% Acetic acid.
A saturated solution of $(NH_4)_2SO_4$ in distilled water.
Dextran (Poviet, Amsterdam), mean molecular weight 200,000, 5% in saline.
Macrodex (Poviet, Amsterdam), a dextran, mean molecular weight 75,000, 6% in saline.

The following biological reagents were used:

Heparin (Vitrum, Stockholm), 1 ml = 5000 I.U.
Serum of a donor with blood group AB, who was apparently

healthy, had not been pregnant, and had never received a blood transfusion. This serum will be called AB serum.
A 20% solution of bovine albumin (Poviet, Amsterdam).
The other biological reagents will be described below.

To facilitate microscopical readings of the leucocyte agglutination reaction, the following device was constructed. From ordinary glass a large microscope slide, 50 x 300 mm, was made. Lines were cut in the glass with a glass-knife, dividing it into 20 equal rectangles. The ordinary object-holder of the microscope was removed and replaced by a light-metal strip, 130 x 70 mm, with a central open rectangle of 30 x 50 mm. Two springs made it possible to secure the large microscope slide, lightly clamped, on the metal strip. In this way the use of the mechanical stage was still possible, while 20 samples could be read without changing the slide (fig. 1).

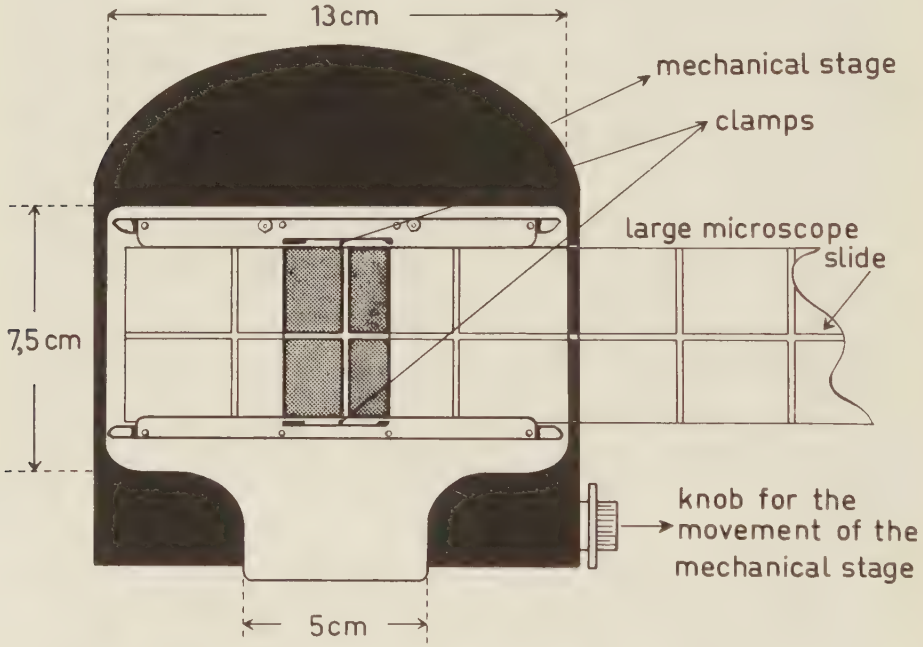

Figure 1
Bird's eye view of the device facilitating the microscopical reading

Sera

The sera from pregnant women were obtained from the Departments of Obstetrics of the University Hospital, Leyden (head, Prof. Dr. A. J. M. Holmer) and of the St. Elisabeth Hospital in Leyden (Dr. R. T. P. Niemer). In all more than 2000 sera were examined. Serial numbers

were given to the women whose serum proved to contain leucocyte agglutinins.

Sera from 520 patients - men, and women who had never been pregnant - were obtained from other departments of the University Hospital. This group is called the group of non-pregnant patients. Most of these sera were obtained from patients of the Department of Cardiology, who were eligible for open-heart surgery. A group of 68 sera from patients who had suffered from a transfusion reaction was also investigated.

In addition the sera from 545 donors of the Blood Transfusion Service were examined.

Blood was obtained by a smooth venepuncture and was collected in a dry clean tube or bottle. No special measures were taken to secure aseptic conditions. After incubation for at least one hour at 37°C the blood was centrifuged and the serum transferred to a dry clean tube. The sera, if not examined the same day, were stored at -20°C after addition of 10% sodium azide solution (one drop per 8 ml serum) and were inactivated at 56°C for 30 minutes before use. If the sera were not clear after storage or a manipulation e.g. absorption, they were centrifuged at 16,000 rpm (16,000 g) for 10 minutes at +4°C.

Leucocyte suspensions for the agglutination reaction

The blood for the agglutination and absorption reactions was in general obtained from the so-called panel, consisting of a group of 100 persons with bloodgroup O: nurses, doctors and technicians working in the University Hospital. They were numbered at random.

For the estimation of gene frequencies and the screening of sera for the presence of leucocyte agglutinins blood samples from donors of the Blood Transfusion Service were used.

Blood has also been obtained from women who had formed leucocyte agglutinins, their husbands and children. Finally the blood from 23 randomly selected families was collected.

The three cell suspensions to be described in the following paragraphs were used within two hours of the venepuncture.

Preparation of the leucocyte suspension from defibrinated blood

Blood was obtained by clean venepuncture and about 20 ml was collected in a siliconised 50 ml conical flask containing approximately 20 glass beads. The blood was defibrinated by an ellipsoid movement ($\pm$ 80 rotations per minute) of the bottle. Foaming was avoided as much as possible. The defibrinated blood was decanted into a siliconised tube type C and mixed with 2 ml dextran solution and 1 ml Tween 80 solution. After incubation of the tube at 37°C for about 30 minutes at an angle of 45° the upper four-fifths of the supernatant plasma was transferred to a siliconised centrifuge tube with a siliconised pipette. This part of the plasma contained 4000 $\pm$ 1000 leucocytes per mm^3 and was used in the agglutination reaction.

If necessary leucocyte suspensions were concentrated by centrifuging the suspension for 10 minutes at 1000 rpm (120 g) in siliconised centrifuge tubes. If a suspension of e.g. 40,000 leucocytes/mm^3 was

required and the original suspension contained 4000 leucocytes/mm^3; 9/10 of the supernatant was removed and the sediment was resuspended in the remaining plasma.

Preparation of the leucocyte suspension from EDTA blood

Blood was obtained by a clean venepuncture and about 8 ml was collected in a siliconised centrifuge tube containing 1 ml EDTA solution. After mixing the EDTA blood was poured into a siliconised tube type C containing 2.5 ml dextran solution. The tube was incubated at 37°C for 30 minutes at an angle of 45°. After incubation the upper four-fifths of the supernatant plasma was transferred with the aid of a siliconised pipette to a siliconised centrifuge tube. This part of the plasma contained 6000 $\pm$ 1500 leucocytes per mm^3 and was used for the agglutination reaction.

Leucocyte suspensions with a higher concentration were prepared as described in the preceding section.

Preparation of "pure" granulocyte and "pure" lymphocyte suspensions

Blood was obtained and a leucocyte suspension was prepared as described in the preceding section. The leucocyte suspension was centrifuged at 1000 rpm for 10 minutes. The platelet-rich plasma was removed and the sediment resuspended in three drops of the same plasma. This concentrated leucocyte suspension was cautiously and slowly squirted into 1 ml saline, which had been layered above 2.5 ml of bovine albumin in a siliconised centrifuge tube. This tube was centrifuged at 3000 rpm (1000 g) for 15 minutes. All erythrocytes and the greater part of the granulocytes were thereby concentrated in the sediment, while all the remaining platelets and the greater part of the lymphocytes occupied a layer between the albumin solution and the saline. The amount of contamination, i.e. granulocytes in the lymphocyte suspension and vice versa, varied from one sample to another. About 75% of the suspensions contained less than 5% contamination with the other component; only these suspensions were used.

Cell suspensions for the absorption experiments

Leucocyte suspensions for cross-absorption experiments

These suspensions were prepared as described under "Preparation of the leucocyte suspension from EDTA blood". "Packed" leucocytes were used for the absorption i.e. the leucocyte suspension was centrifuged for 30 minutes at 3000 rpm and the plasma was removed. The cells were not washed in saline. The cell suspensions contained not only leucocytes, but also erythrocytes and platelets. The erythrocytes do not contain the leucocyte iso-antigens under study, but the platelets do (see chapter four). As this antigen is present on the platelets only if it is also carried by the leucocytes, the contamination of the cell suspension with platelets did not interfere with the group specificity of the cell suspension. The average suspension contained 100 to 300 erythrocytes and 2000 to 10,000 platelets per 100 leucocytes. An average of 1.8×10^8 leucocytes could be isolated from 50 ml of blood.

"Pure" leucocyte suspensions

Blood was obtained by a clean venepuncture and collected in a bottle containing approximately 100 I. U. of heparin per 10 ml of blood. The heparinised blood was centrifuged for 15 minutes at 1000 rpm (120 g). The supernatant platelet-rich plasma was transferred to a second siliconised centrifuge tube (tube B) and the sediment of erythrocytes and leucocytes was put aside (tube A). The platelet-rich plasma (tube B) was centrifuged for 45 minutes at 3000 rpm. The sediment of erythrocytes and leucocytes (tube A) was resuspended in the platelet-poor plasma so obtained and the whole procedure was repeated. Three volumes of platelet-poor blood were mixed with one volume of Macrodex and the mixture was incubated for 45 minutes at 37°C. The resulting platelet-poor leucocyte suspension was centrifuged during 15 minutes at 1500 rpm, the supernatant plasma was discarded and the cellular sediment was resuspended in saline. Due to the paradoxical sedimentation (48) a leucocyte suspension was obtained with approximately one erythrocyte and two platelets per 100 leucocytes. Similar results can be obtained by differential centrifugation of the leucocyte suspension instead of the whole blood. Packed leucocytes were used for absorption.

Thrombocyte suspensions

These were obtained from EDTA blood by differential centrifugation as described above. The platelet sediments were washed twice in saline. They contained less than two erythrocytes and less than one leucocyte per 100 platelets.

The cell suspensions described above were used within four hours after the venepuncture.

Erythrocyte suspensions

The blood was obtained and processed as described under "Preparation of the leucocyte suspension from EDTA blood". To obtain a pure red-cell suspension the supernatant plasma was removed after dextran-sedimentation together with the upper 1/10 of the erythrocyte sediment. After the addition of 2.5 ml of dextran solution to the erythrocyte sediment the blood was once more incubated for 30 minutes at 37°C and the supernatant plasma with the upper 10% of the red cell sediment was again removed. The erythrocyte sediment contained not more than 4% of the leucocytes originally present. This sediment was washed three times in saline, the upper part of the "packed cells" being removed after every washing. The resultant concentration of leucocytes in the final erythrocyte suspension was less than 1% of the leucocytes originally present.

Preparation of placental and kidney tissue for absorption tests

Ten to forty grams of the (membrane-free) fresh tissue were cut into small blocks, washed free of blood in saline and homogenised in a Waring blender at 0°C ($2\frac{1}{2}$ minutes at full speed). The tissue was washed by centrifuging in saline until the washings were no longer pink. The resulting sediment was used for the absorption tests. From

an adjacent part of the tissue histological sections were prepared. The results of the absorption experiments were considered to be representative of the antigenic structure of the tissue under study only if the histological sections were free or practically free from leucocyte infiltration.

Agglutination reactions

With leucocytes from defibrinated blood (Dausset's test (20))

Two drops (equal to approximately 0.10 ml) of a leucocyte suspension were mixed in non-siliconised rhesus tubes with two drops of inactivated serum or serum dilution. The tubes were then incubated for 120 minutes at 37°C. After the incubation the supernatant plasma was removed and one drop of 6% acetic acid was added to the sediment to lyse the red cells. The sediment was transferred to a non-siliconised slide and read microscopically (magnification 50 - 100 x). When the serum and the blood used for the leucocyte suspension were incompatible with regard to the ABO groups, the anti-A or anti-B was absorbed out of the serum with leucocyte-poor A_1 or B packed erythrocytes before the serum was used in the leucocyte agglutination reaction.

Titration of the serum was performed by stepwise twofold dilution in normal AB serum. In comparative titrations of one serum against different leucocyte samples or antigen concentrations the serum under study was titrated in "bulk". This was done as follows: if eight identical titrations of a serum A were needed one ml of serum A was titrated by stepwise twofold dilution in AB serum. Hereafter two drops of leucocyte suspension were mixed with two drops of serum A or of the dilution of serum A obtained as mentioned above. In this way differences between the rows of dilutions are reduced to a minimum.

The following code was used in scoring the agglutination patterns: - (negative) =0, weak or dubious = 1, + = 2, ++ = 3, +++ = 4, ++++ = 5 (see fig. 2*). A cell suspension in which the leucocytes were evenly distributed was denoted negative. Macroscopically visible clumps with almost no free cells were graded as ++++. Between these two extremes, the reaction was scored according to the size of the clumps and the number of free cells. The sum of the numbers given to the results of a titration is the total score of the titration.

A serum was considered to contain leucocyte agglutinins only if leucocyte agglutinins could be demonstrated in more than one serum sample procured on different days and if these agglutinins gave a two plus reaction with at least one leucocyte sample, or if the serum was able to agglutinate the leucocytes of the husband in a titre of at least 2.

With leucocytes from EDTA blood (van Loghem's test (44))

This procedure was identical with the one described in the preceding section except for the difference that EDTA was used to make the blood incoagulable. Moreover the titrations were carried out in saline

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* Tables and figures marked with an asterisk are to be found at the end of this publication.

instead of AB serum. The different leucocyte agglutination patterns are shown in fig. 3*.

With both procedures the test was done in duplicate and recorded as the mean of the two observations. The reactions performed during the screening of the sera for the presence of leucocyte agglutinins were, however, carried out only once with each leucocyte sample.

Absorption tests

With leucocyte, thrombocyte and erythrocyte suspensions

Packed cells were incubated for 30 minutes at 37°C in various known concentrations with a known amount of inactivated serum containing antibodies against leucocytes. After centrifugation of the mixture at 3000 rpm for 45 minutes, two drops of the absorbed serum were titrated against the leucocytes of the donor of the cell suspension which had been used for the absorption. As a comparison the non-absorbed serum was also titrated against the same leucocyte suspension. In cross-absorption tests the absorbed serum was also tested with the leucocytes from other donors. To exclude the possibility that the absorption of the agglutinin was caused by a non-specific effect, the serum was absorbed not only with cells which carried the corresponding antigen, but also with cells which did not carry this antigen.

With placental and kidney tissue

Leucocytes from the patient whose tissue was under investigation were tested with about 12 sera, which contained agglutinins against leucocytes and which had been obtained from other patients. A serum which gave a strongly positive reaction with the leucocytes from the patient was used for the absorption tests (serum A). In order to exclude the possibility that the absorption was brought about by a non-specific factor, another serum with antibodies which reacted negatively with the leucocytes of the patient was also absorbed with the tissue under study (serum B).

The sera used for the absorption tests with placental tissue were selected after they had been incubated not only with the leucocytes of the mother but also with the leucocytes of the child, as it is impracticable to separate the maternal from the foetal part of the placenta. Serum A agglutinated the leucocytes from both the child and the mother, while serum B did not agglutinate either leucocyte sample.

The absorption was performed as follows: 0.8 ml "positive" serum A and 0.8 ml "negative" serum B were each incubated with approximately 0.7 gram (wet weight) of homogenised and washed tissue sediment. After carefully resuspending the sediments the two mixtures were placed in the waterbath at 37°C for 30 minutes. They were then centrifuged at 16,000 rpm (16,000 g) for 10 minutes at +4°C. The sera were tested before and after absorption with the leucocytes from the patient (or, in the case of placental tissue, the mother and the child) and those from a random donor who reacted positively with serum B.

Elution tests

Weiner's method was used (76): leucocytes which had been used for an absorption experiment (2.5×10^8 leucocytes with 1 ml serum) were washed thrice in saline. The leucocytes were lysed by freezing at -20°C after the supernatant saline had been removed.

Ethanol 50% v/v cooled to -20°C, a volume of 10 times the original volume of the packed leucocytes, was added to the lysed leucocytes after thawing. The cell ghosts were resuspended in the ethanol and the suspension was incubated at -20°C for at least one hour. After 5 minutes centrifugation at 3000 rpm the supernatant was removed and the sediment was resuspended in distilled water. After another centrifugation the sediment was resuspended in saline, and the tube was incubated at 37°C for 60 minutes. The supernatant obtained after a final centrifugation is the "eluate".

Isolation of γ-globulin

Kendall's method was used (33): 5 ml of a saturated $(NH_4)_2SO_4$ solution was added to 10 ml serum. The sediment was dissolved in 5 ml distilled water. Two and a half ml $(NH_4)_2SO_4$ solution was added with constant stirring to this solution. The procedure was repeated five times. The resulting euglobulin fraction was dialysed against saline until free of sulphate. The water-insoluble euglobulin fraction was precipitated by 1 - 2 hours' dialysis at +4°C against four changes of distilled water. The supernatant obtained after centrifugation contained the γ-globulins.

Calculations

To establish the correlation between two variables Fisher's 2 x 2 test was used (32).

As described in chapter four 66 sera were tested against the leucocytes from EDTA blood of a panel of 100 random donors (Table VI*). Each vertical row shows the results obtained with one serum. It was necessary to compare the results of each serum with those of all other sera. This implied that more than 2000 times two rows of results, each consisting of 100 agglutination tests, had to be compared. The results originally consisted of positive, negative and doubtful results. To facilitate the calculations the dubious results were changed in "positive" or "negative" according to the following rule: if serum incubated with leucocytes from EDTA blood gave a dubious reaction, the reaction was recorded as positive if the same serum leucocyte combination gave a positive reaction with leucocytes from defibrinated blood. If the serum-leucocyte combination gave a negative reaction with leucocytes from defibrinated blood the reaction was recorded as negative. To compare the two vertical rows of agglutination tests the leucocyte samples were divided into four groups:

group A consisted of those leucocyte samples which gave negative results with the two sera (the number of leucocyte samples belonging to group A is called a);

group B consisted of those leucocyte samples which gave a negative result with the first serum and a positive result with the second serum (= b);
group C consisted of those leucocyte samples which gave a positive result with the first serum and a negative result with the second serum (= c), while group D consisted of the leucocyte samples which were agglutinated by both sera (= d).

In view of the large number of comparisons to be made the statistical sorter IBM 101 was used for the classification of the results into the above-mentioned groups.

In order to investigate to what extent the distribution of the leucocyte samples in the above-mentioned groups could be due to chance Fisher's 2 x 2 test was used (32). The necessary calculations were made by the electronic calculator IBM 604 using the following formula:

$$\chi^2 = \frac{(ad - bc)^2 \ (a + b + c + d)}{(a + b) \ (c + d) \ (a + c) \ (b + d)}$$

The resulting χ^2 values were tabulated in two written records: in the first place in the order of the serum numbers used in the comparison e.g. serum no. 1 with sera nos. 2, 3, 4, 5 etc. (Record A) and in the second place in the order of decreasing magnitude of the χ^2 values (Record B).

The gene frequencies were calculated as follows (67): assume a theoretical leucocyte group Z has the phenotypes Z^a, Z^aZ^b, and Z^b. For Z^a the genotype frequency will be:

gene Z^a = frequency phenotype Z^a + $\frac{\text{frequency phenotype } Z^aZ^b}{2}$

and for

gene Z^b = frequency phenotype Z^b + $\frac{\text{frequency phenotype } Z^aZ^b}{2}$

After the estimation of the gene frequencies the expected frequencies of the phenotypes Z^a, Z^aZ^b and Z^b (in percentages) can be calculated as follows:

expected phenotype Z^a = (gene frequency Z^a)^2 x 100;
expected phenotype Z^aZ^b =
(gene frequency Z^a) (gene frequency Z^b) x 2 x 100;
expected phenotype Z^b = (gene frequency Z^b)^2 x 100.

A direct comparison of the expected and the observed distribution of the phenotypes will in many cases give an impression of consistency. To confirm this impression Fisher's χ^2 test for MN results can be used (67)

$$\chi^2 = \frac{((Z^aZ^b)^2 - (4 \times Z^a \times Z^b))^2}{(2 \times Z^a + Z^aZ^b)^2 \ (Z^aZ^b + 2 \times Z^b)^2}$$

in which Z^a, Z^aZ^b and Z^b are the observed numbers. This test can only be used if the genes are di-allelic.

Linkage

The presence of linkage was investigated with Smith's method (70) using Morton's tables (52). The procedure used can best be explained with the help of an example. In figure 4 the distribution of the hypothetical leucocyte group Z and the blood group MN in an imaginary family is depicted. We want to know what information this family can

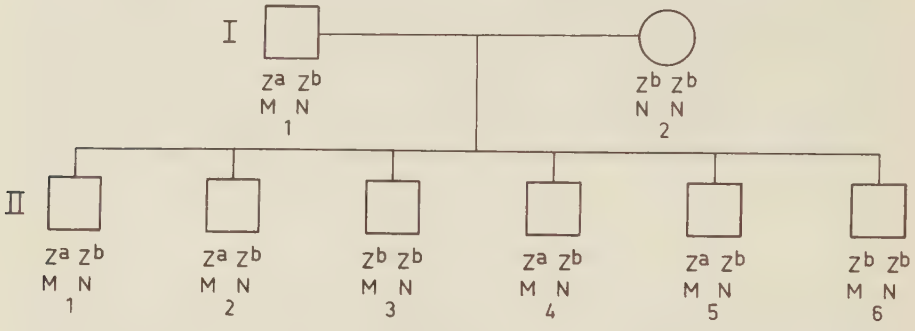

Figure 4

Pedigree of a hypothetical family

give on the existence of linkage between the leucocyte group Z and the blood group MN. The mother must inevitably give to all her children Z^b and N. The father's contributions, however, give evidence on the linkage (at some value θ) or non-linkage (independent assortment) of the Z and MN loci. This mating is tabulated in Morton's table 4 (52) as mating type 6. The value a from this table is in the family considered = 0, b = 2 (children nos. 3 and 6), c = 0 and d = 4 (children nos. 1, 2, 4 and 5), while s = 6.

The probability ratio for a particular θ when transformed to logarithms$_{10}$ is the value "lod" or "z" tabulated by Morton. In this case it is tabulated as z_1 (table 10 of Morton (52)) and this value assumes that the two alternative arrangements of the 4 relevant alleles in the father (coupling and repulsion) are equally likely. This family has s = 6, a + d = 4; b + c = 2 (or a + d = 2; b + c = 4 which gives the same result).

In the same manner the lod or z_1 scores for the other families can be found. By totalling the z_1 scores over all families the total z_1 scores for each θ are obtained. The antilogs of the lod scores are the relative probabilities. Λ is the average of the odds between $\theta = 0.0$ and $\theta = 0.5$ while Λ_2 is the average between $\theta = 0.0$ and $\theta = 0.2$. The final odds on linkage can be calculated by multiplying Λ by 1:21 which is the a priori odds that two genes are lying on the same chromosome. Using Ford's figure of 3000 centimorgans for the total chromosome length of man (27), the a priori chance of a locus being within 20% recombination fraction (say 27 centimorgans) of any other marker is $\frac{27 \times 2.}{3000}$ The factor 2 was introduced because the locus may be on either side of the marker. By combining these odds with Λ_2 the final odds on linkage closer than 20% can be calculated.

Chapter three

THE AGGLUTINATION REACTION

VARIABLES WHICH INFLUENCE THE OUTCOME OF THE LEUCOCYTE AGGLUTINATION REACTION

Introduction

The leucocyte agglutination reaction in approximately the form we have been using was introduced by Dausset et al. (20). Later Colombani and Dausset (17), Killmann (37) and Payne (54), studied the inhibiting effect of a heat-labile substance in serum on the leucocyte agglutination. Colombani and Dausset assumed that this inhibitor is identical with complement. They further assumed that the phenomenon of inhibition is in fact not a true inhibition, but that it is due to lysis of the leucocytes. In their view leucocyte agglutinins can behave in two ways: in the presence of a normal amount of complement lysis of the leucocytes occurs, while agglutination takes place if the complement concentration is lowered. Maximal agglutination occurs if the complement concentration is 1/3 of the normal, i.e. if one part of a leucocyte suspension of defibrinated blood is added to two parts of inactivated serum. Killmann demonstrated (37) that the inhibiting effect is sometimes present in normal serum and on the other hand can be absent from agglutinin-containing sera. As the agglutinins are resistant to heating at 56°C for 30 minutes and this process inactivates the inhibitor, we have used our sera only after such a treatment.

A variable introduced by Killmann was the addition of Tween 80 to prevent non-specific agglutination (37). We were able to confirm his observation and have since used Tween 80 routinely. Killmann (37) confirmed the finding of Dausset (20) that divalent cation binders such as EDTA, oxalate and citrate, and also another anticoagulant, heparin, were able to inhibit the agglutinating properties of some, but not all sera. The mechanism of this phenomenon is not understood.

Payne came to similar conclusions and reported, that non-specific agglutination occurs at the higher dilutions when serum is titrated in saline (54). She advises therefore the use of normal inactivated AB serum instead of saline. We were able to confirm her observation and have followed her suggestion accordingly.

The group of van Loghem (44) introduced an essential modification of the method by using leucocytes obtained from EDTA blood as has also been described by Wasastjerna (75). It soon became apparent that a lower percentage of positive results was obtained with leucocytes from EDTA blood than with leucocytes from defibrinated blood as originally used by Dausset. Engelfriet and van Loghem claimed that this was due to the fact that with leucocytes from EDTA blood fewer non-specific reactions occurred (25).

Dausset (20) and Killmann (37) maintained, however, that EDTA inhibited specific agglutination. No systematic comparison of the two

methods (i.e. using leucocytes from defibrinated and EDTA blood) has been published so far.

Brittingham (10) studied the influence of several anticoagulants on the inhibition of non-specific agglutination and came to the conclusion that EDTA gave the best results. ABO incompatibility between serum and leucocytes from defibrinated blood causes false positive reactions while with leucocytes from EDTA blood this does not occur, as has been pointed out by Lalezari (39). Other variables have also been introduced (dextran instead of polyvinyl pyrrolidone, variation of incubation time and temperature, number of leucocytes in suspension etc.), but none of them has been systematically studied with regard to their influence on the outcome of the agglutination reaction. Table 1* summarises how the test procedure varied from one author to another.

Although, as appears from the above, much work had already been done it was felt that a systematic comparison of the two major versions of the test procedure, i.e. using leucocytes from defibrinated blood and leucocytes from EDTA blood, was essential in order to obtain a better insight into the value and limitations of the two methods. Before this was done three variables, presumably of major importance for the outcome of the test, i.e. the incubation time, the incubation temperature and the number of leucocytes used in the suspension were investigated.

Incubation time

Five sera (nos. 1, 12, 22, 25 and 34) were titrated in bulk and incubated with leucocytes from five donors (nos. 29, 40, 51, 66 and 89). After 30, 60, 90, 120, 150 and 180 minutes' incubation the tests were read. Table II shows the increase of the score when the incubation time is prolonged from 30 to 120 minutes. Thereafter false positive results occur with both techniques. The optimal incubation time is therefore 120 minutes and this period should not be exceeded.

Incubation temperature

Fifteen sera were titrated in bulk and incubated with the leucocytes of five donors at 4°, 22° and 37°C for two hours.

Table III shows that in general a maximal score is obtained after incubation at 37°C when leucocytes from EDTA blood are used. With leucocytes from defibrinated blood the scores obtained at 22°C and 37°C were approximately the same. On the basis of these findings an incubation temperature of 37°C was used for both techniques.

Number of leucocytes in the suspension

Ten sera with leucocyte agglutinins were titrated in bulk and incubated with leucocyte suspensions from seven donors carrying the corresponding antigen. From the blood of each donor at least six leucocyte suspensions, each with a different number of leucocytes per mm^3, were prepared. The number of leucocytes varied from 500/mm^3 to

TABLE II

Relation between incubation time and score

Leucocytes from defibrinated blood (donor no. 29)

		Dilutions of serum no. 1								score
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Incubation time:	30 min.	+	+	-	-	-	-	-	-	4
	60 min.	+	+	(+)	(+)	-	-	-	-	6
	90 min.	+	+	+	(+)	-	-	-	-	7
	120 min.	++	+	+	(+)	(+)	(+)	-	-	10
	150 min.	++	+	+	+	+	+	(+)	(+)	15
	180 min.	++	+	+	+	+	+	+	+	17

Dilutions of serum no. 22

		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Incubation time:	30 min.	-	-	-	-	-	-	-	-	0
	60 min.	-	-	-	-	-	-	-	-	0
	90 min.	-	-	-	-	-	-	-	-	0
	120 min.	-	-	-	-	-	-	-	-	0
	150 min.	+	+	+	+	+	+	+	+	16
	180 min.	+	+	+	+	+	+	+	+	16

Leucocytes from EDTA blood (donor no. 29)

		Dilutions of serum no. 1								score
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Incubation time:	30 min.	-	-	-	-	-	-	-	-	0
	60 min.	++	+	+	-	-	-	-	-	7
	90 min.	+++	++	++	+	(+)	-	-	-	13
	120 min.	++++	++++	+++	+++	++	+	(+)	-	24
	150 min.	++++	++++	+++	++	++	+	+	+	26
	180 min.	++++	++++	+++	++	++	++	++	++	29

Dilutions of serum no. 22

		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Incubation time:	30 min.	-	-	-	-	-	-	-	-	0
	60 min.	-	-	-	-	-	-	-	-	0
	90 min.	-	-	-	-	-	-	-	-	0
	120 min.	-	-	-	-	-	-	-	-	0
	150 min.	+	+	+	+	+	+	+	+	16
	180 min.	++	++	++	++	++	++	++	++	24

TABLE III

Relation between incubation temperature and score

Leucocytes from defibrinated blood						
from donor no:		9	9	9	25	66
with serum no:		10	12	18	22	133
		score				
Incubation	4°C	0	3	1	2	0
temperature:	22°C	2	13	6	18	14
	37°C	4	14	4	16	14

Leucocytes from EDTA blood						
from donor no:		9	9	9	25	66
with serum no:		10	12	18	22	133
		score				
Incubation	4°C	0	0	0	2	0
temperature:	22°C	0	4	3	12	11
	37°C	3	12	6	17	16

more than 24,000/mm^3. As a negative control a compatible serum-leucocyte suspension was used as well as normal AB serum.

Figure 5 shows a typical experiment demonstrating that with leucocytes from defibrinated blood false positive results occur if the suspension contains 6000 or more leucocytes/mm^3. The score is higher when 4000 leucocytes/mm^3 instead of 2000 leucocytes/mm^3 are used. The agglutination pattern obtained with leucocyte suspensions containing more than 10,000 leucocytes per mm^3 differs from that obtained with leucocyte suspensions having a lower number of leucocytes per mm^3 in that in the former the agglutinates are surrounded by a larger number of free cells.

Figure 6 depicts the results of a similar experiment using leucocytes obtained from EDTA blood. To facilitate comparison the score is expressed as a percentage of the maximal score. In this experiment false positive results did not occur. The highest score was obtained with leucocyte suspensions having 8000 leucocytes per mm^3. Table IV shows however that the concentration of the leucocyte suspension which gave a maximal score varied from 2500 to 18,000/mm^3 for the different serum-leucocyte combinations.

In this connection it is important to realise that the normal leucocyte suspension from EDTA blood contains 6000 ± 1500 leucocytes per mm^3.

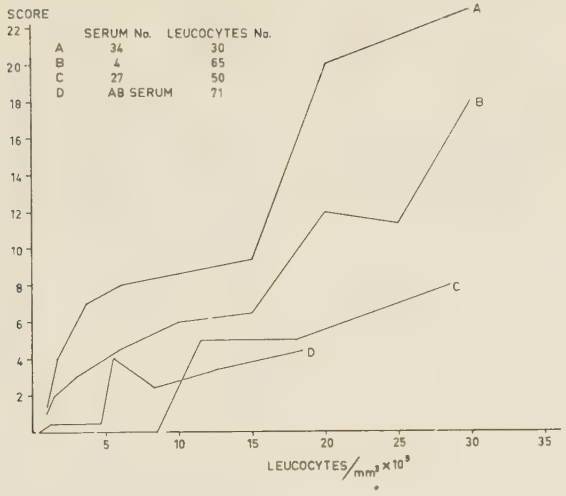

Figure 5

Relation between number of leucocytes per mm^3 in the cell suspension and the score obtained using leucocytes from defibrinated blood (the serum-leucocyte combinations A and B were incompatible ; the combinations C and D were included as negative controls)

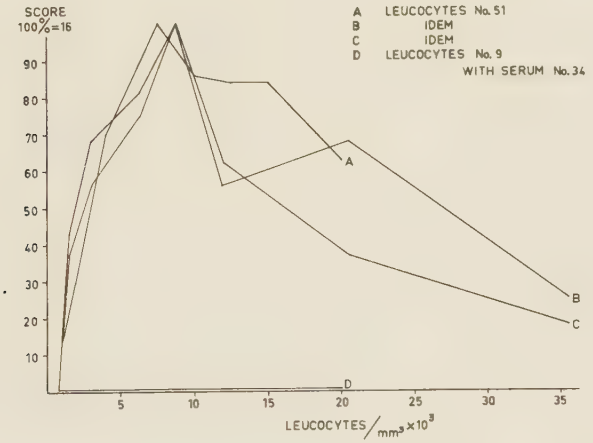

Figure 6

Relation between number of leucocytes per mm^3 in the cell suspension and the score obtained using leucocytes from EDTA blood (the serum-leucocyte combinations A, B and C were incompatible, combination D served as a negative control)

TABLE IV

Correlation between number of leucocytes per mm^3 and maximal score

serum no.	leucocytes from donor no.	concentration of leucocytes per mm^3 for maximal score		
		minimal	maximal	mean
34	51	7500	9000	8000
110	45	5000	11000	9000
1	51	2500	17500	10000
4	51	8000	12500	10000
7	66	9000	11000	10000
50	51	9000	12000	11000
133	45,79	6000	16000	11000
22	26,79	6000	18000	12000
23	9	10000	14000	12000
42	96	11000	16000	14000

Defibrinated versus EDTA blood

Leucocyte samples from defibrinated and EDTA blood have been prepared from 100 random donors. These samples were tested against 62 sera with leucocyte agglutinins formed as a result of pregnancy (tables V* and VI*).

Table VII* shows the number of doubtful reactions obtained with the two techniques. In every instance the number of dubious results was greater with Dausset's test.

For 54 of the 62 sera the number of positive results with Dausset's test is higher than with van Loghem's test. This was also the case if the doubtful reactions were recorded as negative.

The sera could be divided into three groups: 23 sera which gave in 70% or more of the tests identical results with the two methods; 6 sera (nos. 17, 49, 52, 60, 62 and 63) with negative or almost negative reactions in van Loghem's test, but positive in Dausset's test; and the remaining 33 sera, which gave with both tests less than 70% identical results, Dausset's test in all instances being more often positive.

Reproducibility

Ten sera were tested with leucocyte samples from defibrinated and EDTA blood obtained from 10 donors. The same sera were re-tested with fresh leucocyte samples from the same donor on a different day. Table VIII shows the reproducibility of the results, which was poor in

TABLE VIII

Reproducibility of the leucocyte agglutination reaction using leucocytes from defibrinated and EDTA blood

		Defibrinated blood Results of first test			EDTA blood Results of first test		
		Neg.	Dub.	Pos.	Neg.	Dub.	Pos.
Results of second test	Neg.	10	11	6	41	2	3
	Dub.	9	8	9	5	2	6
	Pos.	2	7	38	0	2	39
	Total	21	26	53	46	6	48

both methods, but statistically significantly better with van Loghem's test than with Dausset's test (table IX, $p < 0.001$). The sera used in this experiment were chosen at random. Sera selected for good reproducibility can give reproducible results up to 95%.

TABLE IX

Comparison of the reproducibility of the agglutination test using leucocytes from defibrinated blood versus the test using leucocytes from EDTA blood

	Results reproducible	
	yes	no
Leucocytes from defibrinated blood	56	44
Leucocytes from EDTA blood	82	18

$\chi^2 = 15.8$ $p < 0.001$

Discussion and conclusions

It is evident from tables II and III that false negative results can occur when the incubation time is shorter than two hours or the incubation temperature lower than 37°C, the latter especially with van Loghem's test. Some serum-leucocyte combinations are, however, maximally agglutinated after less than two hours and give equally high scores at 22°C and 37°C. The possibility cannot be excluded that for other serum-leucocyte combinations maximal reactions are not found at 37°C but at a lower temperature. A systematic investigation of this possibility has not been performed.

It could be argued that what is designated as a false positive result is in reality brought about by a specific antibody, which is so weak

that it only manifests itself after an incubation time of more than two hours. The rebutting argument that this is also seen when a normal AB serum is used cannot be accepted as conclusive because naturally occurring antibodies could be responsible. Unequivocal proof that the "false positive" reaction is correctly classified as such is given by the observation (table II) that the resulting agglutination is unrelated to the amount of serum present in the tube i.e. is just as strong in the highest dilution as in the lowest. When the leucocytes are incubated with saline, spontaneous agglutination can also occur after more than two hours' incubation even when they were procured from EDTA blood.

It is clear from the experiments described above that our test conditions using leucocytes from EDTA blood as described in chapter two are not optimal, because the number of leucocytes in the suspension is often not high enough. Nevertheless we did not change our test conditions for the following reasons. In the first place, the results before and after the change would not be comparable. Secondly table IV shows that the optimal number of leucocytes varies considerably from one serum to another. For that reason it is impossible to use a number of leucocytes which is optimal for all sera. Thirdly even with this test procedure sera with good agglutinating ability can be found.

The data described confirm and extend the reports in the literature. Dausset's test is more often positive, gives more dubious results and is less reproducible than van Loghem's test. The important question whether the increase in positive results is a real gain or whether it is caused by a greater number of false positive results remains unsettled. The following arguments are in favour of the theory that it is a real gain.

1) Leucocyte agglutinins can cause transfusion reactions. In about 50% of cases only the reaction with Dausset's test is positive (65).
2) Bosch at al. have demonstrated that platelets of a donor whose leucocytes are agglutinated by the serum of a patient have a shortened life span when they are transfused into that patient (5). This also holds true if only Dausset's test is positive.
3) If the reason for a serum's being positive only with Dausset's test were to be attributed to false positive agglutination, it is difficult to understand why the agglutination pattern should follow the pattern of leucocyte group Four (65).
4) In an experiment in which homograft skin transplantations were performed leucocyte agglutinins were first not demonstrable. After a booster injection of platelets obtained from the skin donor, leucocyte agglutinins appeared. Dausset's test was the first to become positive while van Loghem's test became positive about five days later (64).

From the above considerations it follows that Dausset's test is to be preferred if one wishes to know whether a serum contains leucocyte agglutinins. To study the antigenic structure of the leucocyte van Loghem's test is more suitable because of its better reproducibility. For that reason only this test was used in the experiments described in chapter four.

The mechanism by which Dausset's test gives more positive results is not known. Three possibilities should be kept in mind. In the first place EDTA could block the action of an agglutinin, which is dependent for its manifestation on complement or an unknown substance which also requires divalent cations (17, 37). In the second place it is pos-

sible that the disappearance of leucocyte bound fibrinogen alters the leucocyte antigen sites, e.g. by making them more easily accessible to the agglutinin (68). Thirdly, both mechanisms could be effective.

It should also be remembered that a difference in the agglutination pattern might occur because a serum contains leucocyte agglutinins of two different specificities, one of which is active only in Dausset's test while the other gives identical results with both techniques.

Finally the problem of prozone should be mentioned. Dausset and Killmann are of the opinion that in an agglutination reaction using leucocytes from defibrinated blood the serum should be tested not only undiluted but also diluted 1/2, 1/4 and 1/8 (21, 38). This is rarely necessary in our experience, if the number of leucocytes in the suspension is at least about $4000/mm^3$ (see table I*).

The influence of the leucocyte groups on the outcome of the agglutination reaction will be discussed in the following sections.

IMMUNOLOGICAL AND OTHER PROPERTIES OF LEUCOCYTE AGGLUTININS

Introduction

Dausset was the first to study in extenso the properties of leucocyte agglutinins formed after immunisation by multiple blood transfusions (20). He could show that these agglutinins are γ-globulins, can be absorbed on leucocytes and eluted from them, are stable at -20°C for several months and at +65°C for 10 minutes, but are destroyed after heating at this temperature for 30 minutes. These iso-immune leucocyte agglutinins have group-specific properties. His findings have been confirmed by other workers (1, 38, 49). Some sera were shown to be able to fix complement (16, 50). Van Rood et al. showed that leucocyte agglutinins formed during pregnancy have the same properties (60). In the following the relevant experiments will be reviewed.

Group specificity

In order to find out whether leucocyte agglutinins formed during pregnancy possess group-specific properties, agglutination reactions using van Loghem's test were performed between sera containing leucocyte agglutinins formed during pregnancy and the leucocytes of four groups of persons, viz.:

a) the women, who had formed the leucocyte agglutinins,
b) their respective husbands,
c) their newborn children, and
d) random donors.

Table X shows that the reaction was consistently negative with the leucocytes of the woman who had formed the leucocyte agglutinins, always positive with the leucocytes of her husband and sometimes positive with those of her child. The number of positive results with the group of random donors varied from 7% to 88%. It thus appears that group specificity does exist.

Location of agglutinin activity in the γ-globulins

γ-Globulin fractions were prepared from sera nos. 5 and 22 by ammonium sulfate precipitation. All the agglutinin activity could be recovered from the isolated γ-globulin fractions.

Absorption tests

"Pure" leucocyte suspensions containing 1×10^7, 2×10^7 and 4×10^7 leucocytes from donor no. 51 were incubated at 37°C for 30 minutes with 0.5 ml of serum no. 12, which was able to agglutinate these leucocytes. Table XI shows that the leucocytes were able to absorb the agglutinin out of the serum and that there is a positive correlation between the decrease in agglutinin content of the serum and the number of leucocytes used for the absorption experiment. This experiment

TABLE X

Demonstration of group specificity of leucocyte agglutinins (van Loghem's test)

Serum no:	Results of agglutination reaction with leucocytes obtained from: serum donor	her husband	newborn child	random donors % pos
1	neg	pos	pos	88
6	neg	pos	neg	20
8	neg	pos	neg	47
9	neg	pos	pos	54
11	neg	pos	neg	11
12	neg	pos	pos	84
14	neg	pos	pos	84
16	neg	pos	pos	66
18	neg	pos	pos	75
21	neg	pos	pos	45
22	neg	pos	pos	65
28	neg	pos	pos	56
33	neg	pos	pos	35
35	neg	pos	pos	29
37	neg	pos	pos	31
38	neg	pos	pos	17
39	neg	pos	neg	61
41	neg	pos	neg	7
43	neg	pos	pos	25
44	neg	pos	pos	9
55	neg	pos	pos	59
56	neg	pos	neg	44
57	neg	pos	pos	27
58	neg	pos	pos	19
71	neg	pos	pos	66
85	neg	pos	pos	18
162	neg	pos	neg	56
167	neg	pos	pos	56
480	neg	pos	pos	79

was repeated more than 10 times, using different serum - leucocyte combinations, with similar results.

Elution tests

It was possible to elute the agglutinin from leucocytes which had been used for an absorption experiment, provided that the titre of the serum was at least 32. This experiment was repeated three times using different serum - leucocyte combinations with identical results. After elution approximately 50% of the agglutinin was recovered.

Titre and avidity

In general the titre was low (2 - 16), with a maximum of 128. It was repeatedly found that a serum would give a higher titre with the

TABLE XI

Absorption experiment

Serum no. 12 with leucocytes from donor no. 51

Leucocyte suspension from donor no. 51 incubated	Dilutions of serum 1/1	1/2	1/4	1/8	1/16
with serum no. 12 before absorption	+++	++	++	+	-
with serum no. 12 after absorption with					
1×10^7 leucocytes	++	+	-	-	-
2×10^7 leucocytes	+	-	-	-	-
4×10^7 leucocytes	-	-	-	-	-

from donor no. 51

leucocytes of one donor than with those of another (table XXXIV). There was no correlation between avidity and the titre of an agglutinin. Some sera with a relatively low titre (e.g. 4) had a very good avidity, while other sera with a higher titre had only a moderate avidity. As was to be expected, sera with a good avidity had in general a low percentage of doubtful reactions.

Temperature sensitivity of the agglutinin

Storage of the sera at -20°C for a maximum of one year caused no significant decrease in titre. Furthermore the agglutinins proved to be resistant to heating at 56°C for 30 minutes. However, after being heated at 70°C for 7 minutes (diluted 1/2 in saline to prevent protein precipitation) they were no longer demonstrable.

Discussion and conclusions

In view of the observations that leucocyte agglutinins formed during pregnancy are located in the γ-globulin fraction of the serum, can be absorbed and eluted, are resistant to heating at 56°C for 30 minutes, do not agglutinate the leucocytes of the woman who had produced the antibody, and show group-specific properties, these agglutinins can be considered to be iso-immune antibodies directed against (an) antigen(s) carried by the leucocytes of the husband. If the leucocytes of the child reacted negatively with the serum of its mother, this implies that the father of the child is a heterozygote (see chapter four). In this case the agglutinins probably have resulted from a previous antigenic stimulation (pregnancies, blood transfusions), as naturally occurring leucocyte agglutinins are very rare (see end of this chapter).

Their properties appear to be identical with those of the antibodies against leucocytes formed after multiple blood transfusions as described by Dausset (20). That they are not identical in all respects has been shown by Engelfriet and van Loghem (25) and by Jensen (31), who

reported that the Coombs consumption test, which is frequently positive after immunisation by blood transfusions, is only rarely positive after immunisation against leucocytes by pregnancy. Some agglutinins formed after multiple transfusions are able to fix complement (16, 50). This has not been systematically investigated as far as agglutinins formed during pregnancy are concerned.

FREQUENCY OF THE OCCURRENCE OF LEUCOCYTE AGGLUTININS

Introduction

It has been demonstrated beyond doubt by Dausset that blood transfusions can cause the formation of leucocyte agglutinins (22). He transfused three groups of two patients each at weekly intervals with 70 ml of citrated blood. Each group of two received the blood of the same donor. Leucocyte agglutinins appeared in 4 of the 6 patients after the seventh injection of blood. This not only proves that leucocyte agglutinins can be formed after blood transfusions, but indicates also that about 7 separate blood infusions are needed for the appearance of the agglutinins. Several authors, particularly André (3) and Payne (54), noted a high degree of correlation between the number of previous blood transfusions received and the percentage of cases in which leucocyte agglutinins were formed. However, in none of these publications had the role of previous pregnancies in the formation of the antibodies been considered. The conclusions reached in these papers, especially with regard to the occurrence of leucocyte agglutinins after a small number of blood transfusions, should therefore be considered not yet definitive.

It has been proved that pregnancy alone also stimulates the formation of antibodies against leucocytes (55, 59). Van Rood et al. were able to show that there is a correlation between the number of previous pregnancies and the percentage of cases in which leucocyte agglutinins are formed. Evidence was also given that in the absence of previous pregnancies leucocyte agglutinins are formed only rarely after less than six blood transfusions (60). The latter observation has been confirmed by Payne (56).

In the following paragraphs more extensive data will be presented on the same problem.

Pregnant women

In total the sera of 2500 pregnant women were investigated for the presence of leucocyte agglutinins. Table XII shows the frequency of the occurrence of leucocyte agglutinins in pregnant women who had not received a blood transfusion, correlated with the number of previous pregnancies. All sera were tested against leucocytes from 10 random donors with the aid of Dausset's test. Table XIII summarises the same kind of data from the sera of another group of women, tested against the leucocytes of 10 random donors with van Loghem's test. The sera from the first group were also tested against 10 random samples with van Loghem's method. In this manner it was possible to compare the number of positive reactions obtained when the sera were tested with 10 leucocyte samples in Dausset's test and van Loghem's test. Only 4 of the 51 positive sera from table XII were negative with van Loghem's test.

TABLE XII

Relation between the number of previous pregnancies and the occurrence of leucocyte agglutinins in a group of pregnant women who had never received a blood transfusion (Dausset's test)

	Number of previous pregnancies:			
	0	1	2 - 3	4 or more
Leucocyte agglutination				
negative	3	92	349	245
positive	0	6	19	26
total	3	98	368	271
% positive	0	6	5	10

general total
740

Pregnant women who had previously received blood transfusions

Tables XIV and XV show that only for one group of women could a sufficient number of cases be collected to permit a valid comparison to be made. This was the group of women with four or more pregnancies. The incidence of leucocyte agglutinins in this group is higher than that in the comparable group shown in tables XII and XIII.

TABLE XIII

Relation between the number of previous pregnancies and the occurrence of leucocyte agglutinins in a group of pregnant women who had never received a blood transfusion (van Loghem's test)

	Number of previous pregnancies:			
	0	1	2 - 3	4 or more
Leucocyte agglutination				
negative	154	121	213	210
positive	0	2	5	14
total	154	123	218	224
% positive	0	2	2	6

general total
719

TABLE XIV

Relation between the number of previous pregnancies and the occurrence of leucocyte agglutinins in a group of pregnant women who had received two or more blood transfusions (Dausset's test)

	Number of previous pregnancies:			
Leucocyte agglutination	0	1	2 - 3	4 or more
negative	2	4	20	28
positive	0	0	3	7
total	2	4	23	35
% positive	0	0	13	20

general total
64

Not only is the incidence of transfusion reactions greater in the group of patients who had been pregnant and had been transfused than in the group which had only received blood transfusions (table XVI), but the incidence of leucocyte agglutinins in the first group is also higher (table XVII).

TABLE XV

Relation between the number of previous pregnancies and the occurrence of leucocyte agglutinins in a group of pregnant women who had received two or more blood transfusions (van Loghem's test)

	Number of previous pregnancies:			
Leucocyte agglutination	0	1	2 - 3	4 or more
negative	2	4	7	27
positive	0	0	2	5
total	2	4	9	32
% positive	0	0	22	16

general total
47

TABLE XVI

Correlation between the occurrence of non-haemolytic transfusion reactions and a history of pregnancy

	Transfusion reaction	
	Yes	No
Previous pregnancy	39	507
No previous pregnancy	29	1231

$\chi^2 = 38.42$ $p < 0.001$

TABLE XVII

Correlation between the presence of leucocyte agglutinins and a history of pregnancy in cases of transfusion reaction

	Leucocyte agglutination (Dausset's test)	
	Pos.	Neg.
Previous pregnancy	27	12
No previous pregnancy	10	19

$\chi^2 = 8.139$ $p < 0.005$

Patients and blood transfusion donors who neither had been pregnant nor had received a blood transfusion

In order to investigate the possible existence of naturally occurring leucocyte agglutinins (i.e. not formed by immunisation of pregnancy or blood transfusion) 225 sera of patients and 50 sera of donors who had not been pregnant and had never received a blood transfusion, were investigated with Dausset's test against leucocyte samples of 5 donors. One sample proved to be weakly positive.

Women with a history of abortion

It is theoretically possible that the presence of leucocyte agglutinins would be harmful for the foetus or might cause abortion. It is also possible that the curettage following an abortion could cause the entrance of foetal material into the blood stream of the mother and in this way stimulate the formation of leucocyte agglutinins. Table XVIII shows, however, that patients with an abortion in their history did not have leucocyte agglutinins in their serum more frequently than women with no such history, but with a comparable number of pregnancies.

TABLE XVIII

Lack of correlation between a history of abortion and the presence of leucocyte agglutinins in pregnant women

Dausset's test

Leucocyte agglutination	History of abortion: Number of previous pregnancies: 0	1	2 - 3	4 or more	No history of abortion: Number of previous pregnancies: 0	1	2 - 3	4 or more
negative	0	49	111	141	3	43	238	104
positive	0	2	7	16	0	4	12	10
total	0	51	118	157	3	47	250	114
% positive	0	4	6	10	0	9	5	9
	general total 326				general total 414			

van Loghem's test

Leucocyte agglutination	History of abortion: Number of previous pregnancies: 0	1	2 - 3	4 or more	No history of abortion: Number of previous pregnancies: 0	1	2 - 3	4 or more
negative	0	28	78	131	154	93	135	79
positive	0	0	3	6	0	2	2	8
total	0	28	81	137	154	95	137	87
% positive	0	0	4	4	0	2	2	9
	general total 246				general total 473			

Formation of antibodies during pregnancy and the persistence of these antibodies after delivery

A group of 37 pregnant women was divided according to the month of pregnancy during which the first screening test on the presence of leucocyte agglutinins was carried out (table XIX). All 37 women formed leucocyte agglutinins. However, the sera of the women investigated during the first five months of their pregnancy contained leucocyte agglutinins significantly less often, than those of the women investigated during the last four months of their pregnancy. The sera which were negative in the first test, became positive at a later date during the same pregnancy. Van Loghem's test was used.

In order to study the persistence of leucocyte agglutinins after delivery serum samples were obtained after delivery from women in whose serum leucocyte agglutinins were found during pregnancy. The presence of leucocyte agglutinins in the absence of further antigenic stimuli could in all cases be demonstrated at least several months after delivery and in one case seven years after delivery.

TABLE XIX

The presence of leucocyte agglutinins in sera obtained during the first or second half of the pregnancy

		First serum sample investigated	
		before the end of the 5th month of pregnancy	after the end of the 5th month of pregnancy
Leucocyte agglutination	pos.	7	14
	neg.	11	5

The presence of Rhesus antibodies in the serum

It has been suggested that the leucocyte agglutinating properties of some sera could have been caused by the presence of Rhesus antibodies in these sera (74). This seems a priori unlikely, since van Loghem's test is not influenced by red cell incompatibility. Conclusive in this respect is the observation that leucocyte agglutinins from sera which also contain Rhesus antibodies are able to agglutinate leucocytes from Rhesus-negative donors (the sera nos. 2, 3, 12, 24, 35, 38 and 51 contain anti-D; for donors who were Rhesus- negative see table VI*).

Discussion and conclusions

Tables XII and XIII show a positive correlation between the number of previous pregnancies and the percentage of women with leucocyte agglutinins. This observation, together with the data discussed in the preceding section (group specificity, antibody properties etc.), allow the statement that these leucocyte agglutinins are iso-immune antibodies directed against an antigen carried by the leucocytes of the father and that they are formed by immunisation during pregnancy or directly after delivery.

It is made evident by the data discussed in the preceding paragraphs that it will be impossible to state in general in what percentage of pregnant women leucocyte agglutinins will be demonstrable, as the percentage will be dependent on the following variables:

1) the technique used: with leucocytes from defibrinated blood more positive sera are found than with leucocytes from EDTA blood;
2) the number of leucocyte suspensions used for the screening: antibodies directed against an antigen with a low frequency will be easily missed unless the serum is tested either with a large number of leucocyte suspensions or with the leucocytes of the husband;
3) the number of previous pregnancies: see tables XII and XIII;
4) the eventual immunisation by previous blood transfusions: see tables XIV and XV;
5) the trimester of the pregnancy in which the blood is investigated: see table XIX.

It is interesting to note that with Dausset's technique the percentage of positive sera found (table XII) was higher than when van Loghem's test was used (table XIII). Nevertheless almost all sera from table XII which were positive proved to be able to agglutinate leucocytes from EDTA blood. This confirmed our findings with another group of sera (table VII*) and illustrates the superiority of Dausset's test over van Loghem's test in answering the question, whether a serum contains leucocyte agglutinins. Moreover this observation proves that the greater percentage of positive results obtained with Dausset's test cannot be solely attributed to a greater number of false positive results.

The apparent contradiction that most of the sera positive with Dausset's test proved to be also positive with van Loghem's test can be explained as follows. A positive result with Dausset's test prompted further investigation of the serum, when it proved in most cases that

1) the leucocytes procured from the husbands were positive in van Loghem's test;
2) an increase in the number of leucocyte samples tested revealed that some gave a positive reaction in van Loghem's test; and
3) a fresh serum sample from the same patient showed agglutination in van Loghem's test.

If during or shortly after pregnancy a woman reacts to a blood transfusion with a transfusion reaction, the chance of finding leucocyte agglutinins is very high (leucocyte agglutinins were found in 10 of 11 such women).

Leucocyte agglutinins which might be naturally occurring were only found once. The agglutinin was weak and only detectable with Dausset's test. As it was still demonstrable in a newly taken serum sample after a year's interval, it seems unlikely that the results were due to false positive agglutination. Auto-agglutination did not occur and the patient, suffering from Gilbert's disease, had a normal number and distribution of leucocytes. The mode of formation of these "naturally" occurring agglutinins is obscure, as is their clinical importance. Their existence has been described by several authors (13, 26, 28, 35, 51, 72, 77) and denied by others (2, 21). Although they appear to exist, their rare occurrence and low titre exclude their use in leucocyte grouping.

It is evident that leucocyte agglutinins can only be produced during pregnancy, if one or more antigens present on the leucocytes of the husband are lacking on the leucocytes of his wife. This implies that a knowledge of the leucocyte groups will permit a prediction of the possibility of agglutinin formation as a result of pregnancy.

Chapter four

LEUCOCYTE GROUPS

LEUCOCYTE GROUP FOUR

Introduction

The arguments in favour of the existence of leucocyte groups have been reviewed in chapter one. The occurrence of four leucocyte groups, the group Mac and the groups Two, Three and Four, has up till now been postulated (22, 59, 60, 63). However, no leucocyte group has been described completely. It is intended to do so in this chapter.

The detection and the description of a leucocyte group offer no difficulties, provided two prerequisites are satisfied. One is a simple and reliable technique and the other is a strong, specific anti-serum, recognising only one leucocyte group, all samples of which will be agglutinated.

In the previous chapter it was shown in detail that our technique, though relatively simple, is not yet sufficiently reliable. It has been argued that, since the reproducibility was better with van Loghem's test than with Dausset's test, the first test should be used for leucocyte grouping. To minimise its shortcomings a sufficiently large number of leucocyte samples must be tested to enable the investigator to employ a statistical approach.

Leucocyte agglutinins formed during pregnancy are to be preferred for grouping, because they have been produced in response to a known and constant source of antigen and therefore less often consist of a mixture of antibodies of different specificity than do leucocyte agglutinins formed after multiple blood transfusions. Cross-absorption cannot rule out the presence of antibodies against different leucocyte antigens, because the serum may contain a mixture of agglutinins of different specificity of which one is directed against an antigen with a very low frequency. Nevertheless cross-absorption can make this unlikely and for this reason the procedure is considered to be essential in leucocyte grouping studies. During such a cross-absorption test our attention was drawn to a pitfall not previously realised. Table XX shows that the leucocyte suspension of donor no. 44 was not agglutinated by serum no. 36, but could nevertheless absorb the agglutinin out of the serum. This phenomenon was reproducible and has also been encountered with other serum-leucocyte combinations. We have named it the agglutination-negative, absorption-positive phenomenon (63). It occurs especially with sera of low or moderate avidity. To decrease the effect of this phenomenon leucocyte grouping should preferably be done with a number of identical sera of high avidity.

The following points apply to every "grouping" study: when it is believed that a leucocyte group has been demonstrated, confirmation should be sought by performing family studies and by looking for the antigen corresponding to the allelic gene.

TABLE XX

Cross-absorption experiment

serum no. 36

		Agglutination reaction of the leucocytes from donor no:											
		38	56	55	50	37	31	51	52	11	44	58	49
with serum no. 36 before absorption:		+++	+++	++	++	++	+	+	+	+	-	-	-
with serum no. 36 after absorption with leucocytes from donor no:	38	-	-	-	-	-	-	-	-	-	-	-	-
	56	-	-	-	-	-	-	-	-	-	-	-	-
	55	-	-	-	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-	-	-	-
	37	-	-	-	-	-	-	-	-	-	-	-	-
	31	-	-	-	-	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	-	-	-	-	-	-	-
	52	-	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-
	44	-	-	-	-	-	-	-	-	-	-	-	-
	58	+++	+++	++	++	++	+	+	+	+	-	-	-
	49	+++	+++	+++	++	++	+	+	+	+	-	-	-

Selection of the sera

Sera were selected from those shown in table VI* . As has been argued above, sera were sought which fulfilled one of two criteria. In the first place more or less identical sera were needed ("more or less" because the technique is limited in its reproducibility) and secondly sera which might recognise the products of allelic genes. If the distribution of leucocyte samples giving positive and negative results with two identical sera is studied as described in chapter two a very high χ^2 -value will be obtained e.g. over 11, corresponding to a probability of less than 1/1000.

Table XXI shows the twenty highest χ^2 -values as they were calculated by the IBM machine 604, after the data had been arranged by the statistical sorter IBM 101 as described in chapter two. To facilitate the interpretation of the large number of these data attention was in the first place focused on the χ^2 -values equal to or greater than 11. It appears that the sera can be divided by means of their χ^2 distribution into four relatively unrelated groups (tables XXII - XXV*). The way this was done can be most effectively explained with the aid of table XXI. The first horizontal row shows that sera nos. 13 and 55 are related (χ^2 -value = 66). On the third row evidence is given that sera nos. 16 and 55 are related. The tenth row demonstrates a relation between sera nos. 13 and 16. Working down the list in this way the tables XXII - XXV* were constructed.

Sera which recognise the products of allelic genes were generally found to be correlated by χ^2 -values smaller than 11. To find such sera the distribution of positive and negative results of a serum belonging to a group of two identical sera (e.g. serum no. 46) were scruti-

TABLE XXI

Data from computer

Record B

First serum :		A neg.	B neg.	C pos.	D pos.	
Second serum:		neg.	pos.	neg.	pos.	χ^2
First serum, serum no: 13	Second serum, serum no: 55	37	5	4	54	66
27	38	75	1	8	16	55
16	55	31	3	10	56	54
14	46	11	5	2	82	52
22	36	30	5	8	57	52
8	28	37	12	5	46	44
22	34	32	3	15	50	43
54	63	93	0	4	3	41
12	18	14	2	11	73	40
13	16	29	13	5	53	40
31	41	84	1	8	7	36
12	48	15	1	16	68	35
26	40	72	4	10	14	35
34	35	46	1	23	30	35
11	38	80	8	3	9	33
13	67	33	9	12	46	33
6	11	77	2	11	10	32
28	55	31	11	10	48	32
35	57	62	7	11	20	32
12	15	12	4	10	74	31

nised (Record A) and sera which agglutinated all or practically all leucocyte samples which were not agglutinated by serum no. 46 were selected to see if their distribution of positive and negative results indicated that they might recognise the product of an allelic gene.

The agglutination pattern of the first group of sera (table XXII) is demonstrated in table XXVI*. The sera nos. 14 and 46 are possibly identical, while the pattern indicates that serum no. 4 might recognise the allele of the gene recognised by sera nos. 14 and 46. This hypothesis finds support in the good correlation between the expected and found frequency of the genes recognised by sera nos. 4 and 46 ($\chi^2 = 0.5$). That even such evidence is not conclusive is demonstrated in table XXVII: cross-absorption showed that serum no. 4 contained a mixture af agglutinins of different specificity.

TABLE XXII

χ^2-values of first group of sera

Second serum, serum no:	4		
	14	10*)	
	46	10*)	52
First serum, serum no:	4	14	46

*) indicates that the corresponding sera might recognise the products of allelic genes.

TABLE XXVII

Cross-absorption experiment

serum no. 4

		Agglutination reaction of the leucocytes from donor no:											
		85	91	86	71	39	53	83	19	34	6	11	80
with serum no. 4 before absorption:		+++	+++	+++	++	+++	+++	+++	+++	+++	-	-	-
with serum no. 4 after absorption with leucocytes from donor no:	85	-	-	-	-	-	-	-	-	-	-	-	-
	91	-	-	-	-	-	-	-	-	-	-	-	-
	86	+	-	-	-	-	-	-	-	++	-	-	-
	71	-	-	++	-	-	-	-	-	++	-	-	-
	39	+	-	-	-	-	-	-	-	+++	-	-	-
	53	+	-	-	-	-	-	-	-	++	-	-	-
	83	++	+	+	-	-	-	-	-	++	-	-	-
	19	++	++	-	-	-	-	-	-	++	-	-	-
	34	-	+++	++	++	+++	++	+++	+++	-	-	-	-
	6	+++	+++	+++	+++	+++	+++	+++	+++	++	-	-	-
	11	+++	+++	++	+++	+++	+++	++	+++	++	-	-	-
	80	+++	+++	++	++	+++	++	+++	+++	++	-	-	-

TABLE XXVIII

Cross-absorption experiment

serum no. 34

		Agglutination reaction of the leucocytes from donor no:													
		25	26	28	27	22	19	70	51	23	17	21	18	24	14
with serum no. 34 before absorption:		++	++	++	+	+	+	++	++	++	-	-	-	-	-
with serum no. 34 after absorption with leucocytes from donor no:	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	27	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	17	+	+	+	+	+	++	+	++	++	-	-	-	-	-
	21	++	++	++	+	++	+++	++	+++	+++	-	-	-	-	-
	18	++	++	+	+	+	++	++	+++	++	-	-	-	-	-
	24	++	+	++	+	++	++	++	+++	++	-	-	-	-	-
	14	++	++	+	+	+	++	++	++	++	-	-	-	-	-

Of the sera shown in table XXIII* twelve were more or less identical, while serum no. 51 might recognise the product of the allelic gene. These sera appeared to be less suitable for further study, because most of them had a low titre and only moderate avidity.

Table XXIV* comprises all the sera which could not be classified in the two previous groups nor in the group shown in table XXV*, which is discussed in the following section.

Leucocyte group Four

Cross-absorption of three of the sera from table XXV*, nos. 12, 34 and 36, demonstrated that in all probability these sera contained antibodies which recognised only one leucocyte antigen.

The results obtained with serum no. 34 are shown in table XXVIII, while the cross-absorption experiment with serum no. 36 has already been given in table XX. The antigen recognised by serum no. 34 had been designated 4^a (the existence of leucocyte groups Mac, Two and Three had already been postulated), and its allele, if any, 4^b (63).

Table XXIX* depicts the agglutination pattern obtained with the twelve sera of table XXV*. Every vertical row shows the results obtained with one serum against the 100 different leucocyte suspensions of the panel. There are four sera more or less identical with serum no. 34 called anti-4^a and seven more or less identical sera called anti-4^b which might recognise the product of a gene allelic to 4^a. Although there are many exceptions the overall results can be divided into three groups. These groups could correspond to the genotypes 4^a4^a, 4^a4^b, and 4^b4^b with a gene frequency for 4^a of 0.38 and for 4^b of 0.62.

The numerous results in table XXIX* which do not follow the general pattern need to be commented on. The majority of the exceptions was found in the results obtained with four sera with a low titre (nos. 19, 35, 50 and 59), one of which (no. 50) proved to contain antibodies against at least two leucocyte groups when examined by cross-absorption. When the leucocytes of the 100 donors were retested with the remaining eight sera the pattern shown in table XXX* was obtained. It is important in this connection to note that on retesting two donors (nos. 17, 85) originally typed as 4^a4^b proved to be 4^b4^b. The exceptions which remained on retesting were probably due to the agglutination-negative, absorption-positive phenomenon.

In addition to the panel shown here another 247 unrelated persons were tested with similar results. The found and expected gene frequencies show good agreement (table XXXI).

Further support for the hypothesis that the sera were recognising a leucocyte group was found in the observation that the leucocytes of the women who had produced the antibody anti-4^a all had the genotype 4^b4^b. Accordingly the leucocytes of their husbands had the genotype $4a4^a$ or 4^a4^b (table XXXII). On the other hand the leucocytes of the women who had produced anti-4^b had the genotype 4a4a, while the leucocytes of their husbands had the genotypes 4^b4^b or $4a4^b$ (table XXXIII). In these tests also some leucocyte samples which were expected to give a positive reaction were not agglutinated. However, these leucocytes were able to absorb the antibody out of the serum.

TABLE XXXI

Expected and observed frequencies of the phenotypes of group Four in a group of 347 random donors

Observed:	numbers	fraction of total
4^a4^a	46	0.1326
4^a4^b	175	0.5043
4^b4^b	126	0.3631
Total	347	

gene freq. 4^a = 0.3847

gene freq. 4^b = 0.6153

Expected:	numbers
$4^a4^a = 0.3847^2 \times 347$	= 51.35
$4^a4^b = 2 \times 0.3847 \times 0.6153 \times 347$	= 164.27
$4^b4^b = 0.6153^2 \times 347$	= 131.37

$\chi^2 = 1.478$ $\quad 0.20 < p < 0.30$

They are indicated in the tables with asterisks.

Further support for the existence of leucocyte group Four was found in the demonstration of a definite dosage effect (table XXXIV). The number of leucocytes per mm^3 was approximately the same in the different leucocyte samples.

TABLE XXXII

Leucocyte groups of the women who had formed anti-4^a, and of their husbands

		anti-4^a					anti-4^b						
	Serum no:	34	36	22	35	50	12	15	18	23	48	59	19
Leucocytes from woman no:	34	-	-	-	-	-	+++	++	+	++	+	***	***
	36	-	-	-	-	-	+++	++	++	+++	++	++	+
	22	-	-	-	-	-	+++	++	+++	++	++	+	+
	35	-	-	-	-	-	++++	+	+	+++	+	***	***
	50	-	-	-	-	-	++++	++	+	+++	++	+	+
Leucocytes from the husband of woman no:	34	+++	+++	++++	++	+++	-	-	-	-	-	-	-
	36	++	+++	++++	***	++	-	-	-	-	-	-	-
	22	+++	++	++++	++	+++	+++	++	++	+	++	+	***
	35	+	++	++	+	++	+++	++	++	+++	++	++	+
	50	+	++	+	+	+++	+++	++	+++	+++	++	+	++

*** agglutination-negative, absorption-positive

TABLE XXXIII

Leucocyte groups of the women who had formed anti-4^b, and of their husbands

		anti-4^a					anti-4^b						
	Serum no:	34	36	22	35	50	12	15	18	23	48	59	19
Leucocytes from woman no:	12	+++	++	+++	+	++	-	-	-	-	-	-	-
	15	+++	++++	++++	+	++	-	-	-	-	-	-	-
	18	+++	+++	++++	++	+++	-	-	-	-	-	-	-
	23	++++	++	++++	+	+++	-	-	-	-	-	-	-
	48	+++	+++	++++	++	++	-	-	-	-	-	-	-
	59	++++	+++	++++	+	++	-	-	-	-	-	-	-
	19	++	+++	+++	++	+++	-	-	-	-	-	-	-
Leucocytes from the husband of woman no:	12	-	-	-	-	-	+++	***	+++	++	++	***	***
	15	-	-	-	-	-	+	+++	+++	++	++	++	***
	18	-	-	-	-	-	+++	++	+++	+++	++	+	++
	23	-	-	-	-	-	++++	++	+	++	+	+	***
	48	-	-	-	-	-	+++	++	+++	+++	++	+	+
	59	++	+++	++++	++	+++	+++	+++	++++	+++	+++	+++	+++
	19	++	+++	++++	++	++	+++	++	++	++	++	++	+

*** agglutination-negative, absorption-positive.

As a final test the distribution of leucocyte group Four in 25 families with 33 matings and 140 children was studied. In twelve instances the results were not absolutely clear-cut and a definitive classification could not be made. This was, however, achieved in 10 of the 12 cases by varying the number of leucocytes in the suspension used for the leucocyte agglutination test and by absorbing one anti-4^a and one anti-4^b serum with the leucocytes of the person involved. After the absorption the sera were retested with 4^a4^a and 4^b4^b leucocytes which were known to give a good positive reaction with these sera. The two children, of which the genotypes are not absolutely certain, were arbitrarily given that genotype which fitted the least well. In the 4 ma-

TABLE XXXIV

Dosage effect

Leucocytes from donor no:		genotype	Dilution of serum no. 34 (anti-4^a) 1/1	1/2	1/4	1/8	score
	51	4^a4^b	++	+	-	-	5
	28	4^a4^a	+++	++	+	-	9
			Dilution of serum no. 12 (anti-4^b) 1/1	1/2	1/4	1/8	score
	51	4^a4^b	++	+	-	-	5
	9	4^b4^b	+++	++	+	-	9

TABLE XXXV

Family studies

MATING		CHILDREN		
		4^a4^a	4^a4^b	4^b4^b
$4^a4^b \times 4^a4^b$	9	10 (11¼)	24 (22½)	11 (11¼)
$4^a4^b \times 4^b4^b$	11	0 (0)	16 (18)	20 (18)
$4^a4^b \times 4^a4^a$	5	6 (9½)	13 (9½)	0 (0)
$4^a4^a \times 4^b4^b$	4	0 (0)	19 (19)	0 (0)
$4^a4^a \times 4^a4^a$	0	- (-)	- (-)	- (-)
$4^b4^b \times 4^b4^b$	4	0 (0)	0 (0)	21 (21)
Total	33	16	72	52
				140

The expected values are given in parenthesis

tings of $4^b4^b \times 4^b4^b$ type all the 21 children were unmistakably of 4b4b type on the results of the first test. No matings of the type $4a4^a \times 4a4a$ were observed (the expected frequency of this mating is 1/46). There existed a gratifying agreement between the expected and the observed distribution of the children's genotypes (table XXXV).

Linkage studies

The following blood groups were determined in the families mentioned in table XXXV: ABO, MNS, P, Rhesus, Kell, Duffy and Kidd (see appendix*). The final chance of linkage closer than 20% was much lower than the initial probability of 0.014, except for Kell and Kidd (table XXXVI). The latter was possibly due to the small number of families studied from which information on these groups was available. The two "uncertain" genotypes had no influence one way or the other on the outcome of the final chance of linkage.

Discussion and conclusions

The evidence presented points to the existence of a leucocyte group and it shows that it is possible by the means used to classify leucocyte samples according to this group.

Because 4^a has about the same frequency as Mac the possibility was entertained that the two are identical. Prof. Dausset was kind enough to test serum no. 22 and serum no. 12 against known Mac-po-

TABLE XXXVI

Data on linkage

Chance of linkage closer than 20% between leucocyte group Four and blood groups:	
ABO	< 0.001
Rhesus	< 0.0001
MNS	< 0.001
P	< 0.001
Duffy	< 0.001
Kell	< 0.022
Kidd	< 0.024

sitive and negative leucocytes (table XXXVII). The results show no significant correlation. It is not possible to state definitively whether anti-Mac and anti-4^a are identical or not, as no reliable anti-Mac serum is available and the number of leucocyte samples typed for Mac is rather small. As a definite conclusion cannot be reached the name "leucocyte group Four" will be retained.

In the selection of sera which recognised leucocyte group Four Fisher's 2 x 2 test was an important item; its application to this problem was rather unconventional. In order to be able to use the 2 x 2 test, it was necessary to record the doubtful results as either negative or positive (chapter two). This was considered permissible for the following reasons:

1) the effect of this method of recording will be proportional to the percentage of doubtful results obtained with a given serum. As sera with a high percentage of doubtful results will be useless in leucocyte grouping anyway the above-mentioned effect will be of no consequence;
2) the χ^2 -values obtained were only used as a means to select sera which possibly could recognise a leucocyte group, and not as definite proof of a positive correlation between two sera.

The last argument can also be used to answer the possible criticism that the formula used to calculate χ^2 -values gives reliable results only if the expected numbers are greater than 5.

TABLE XXXVII

Agglutination pattern of serum no. 34 (anti-4^a) and serum no. 12 (anti-4^b) with Mac-positive and Mac-negative leucocytes (by courtesy of Prof. Dausset)

	Leucocytes of donor:									
	a	b	c	d	e	f	g	h	i	j
anti-Mac	+	+	+	+	+	-	-	-	-	-
anti-4^a	(+)	-	++	++	++	-	-	(+)	-	++
anti-4^b	+	+	+	+	+	++	++	-	++	++

The analysis of the χ^2 -values shows also that antibodies against three leucocyte groups appear fairly often, possibly implying that these groups are more antigenic than other leucocyte groups, of whose existence we are as yet not aware.

The χ^2 -values which could not be classified into the groups shown in tables XXII, XXIII* and XXV* could fall under one of the following headings:

1) they are artefacts, produced by the occurrence of a high percentage of doubtful or negative results or because an expected frequency was lower than 5;
2) they select identical sera or sera which recognise alleles. These were investigated more extensively, if at least three of such sera were found (cf. table XXII). If there were only two of these, they were not further studied;
3) they correlate sera which recognise antigens which are closely linked. The study of linkage is only fruitful when the corresponding leucocyte groups have been fully described;
4) in every 1000 distributions of numbers there will be one which by chance alone shows a distribution of the numbers indicating a correlation significant at the 1/1000 threshold, corresponding to a χ^2 -value of 11.

The fact that even with the precautions taken two leucocyte samples were originally wrongly typed implies that leucocyte grouping is still not completely reliable and that it is necessary to perform it with a number of identical sera.

THE DISTRIBUTION OF LEUCOCYTE GROUP SUBSTANCE

Introduction

The distribution of leucocyte group substance on the different cell types of the leucocyte and on other tissues (as recognised with human iso-immune sera) has been but rarely investigated.

An extensive literature exists on the possibility of differentiating with the aid of heterologous immune sera between lymphocytes and granulocytes (see Walford, 74), but the discussion of the use of heterologous sera lies outside the scope of this study. The evidence on this subject concerning the use of human iso-immune sera is much less convincing. Most of the work has been done with leukaemic cells (2, 11, 21, 36, 38, 50). Although no consistent pattern was observed, the impression was gained that immature leucocytes were less well agglutinated than were mature leucocytes. This conclusion should be regarded with reserve, since leucocyte grouping was not possible at that time. André (1) reports the properties of a serum with leucocyte agglutinins which were able to differentiate between normal lymphocytes and granulocytes; but the general impression is that lymphocytes and granulocytes have iso-antigens in common.

Dausset demonstrated by agglutination and absorption experiments (23) and van Rood et al. by absorption experiments (60) that platelets carry leucocyte group substance. Furthermore van Rood was able to show the presence of leucocyte antigens in placental tissue (59). Chalmers, Coombs, Gurner and Dausset proved with the aid of the mixed antiglobulin reaction that leucocyte antigens were present not only on leucocytes but also on platelets and HeLa cells (15). In the following section relevant observations on the distribution of the leucocyte group substance will be reviewed and new observations will be described.

Granulocytes and lymphocytes

Table XXXVIII* shows that serum no. 4 agglutinated the granulocytes and lymphocytes of donor no. 98, while the granulocytes but not the lymphocytes of donor no. 51 were agglutinated by the same serum. In contrast, serum no. 1 agglutinated only weakly the granulocytes of a random donor, which was given the no. 101, while the lymphocytes of that donor were strongly agglutinated. The agglutination reaction seems not to be influenced by the number of thrombocytes in the suspension.

These findings proved to be reproducible and similar results were obtained with 12 other serum-leucocyte combinations. It thus appears that leucocyte antigens can be found on both granulocytes and lymphocytes, but that the distribution of the antigen varies from one leucocyte sample to another.

Erythrocytes and platelets

The presence of erythrocyte group substance on the leucocyte has been discussed in a large number of articles (see Walford, 74). As

this subject lies outside the scope of this study, these papers will not be discussed here.

The presence of leucocyte group substance on erythrocytes was investigated as follows.

ABO-compatible sera without irregular erythrocyte antibodies but containing leucocyte agglutinins were not able to agglutinate erythrocytes. Furthermore, it was not possible to demonstrate by means of absorption experiments the presence of leucocyte group substance on erythrocytes (table XXXIX).

Platelet agglutination tests have not been performed. In cross-absorption experiments platelets were shown to carry the same antigen(s) as the leucocytes of the platelet donor (table XXXIX). This experiment has been repeated and the results were confirmed in more than 60 other serum-platelet combinations.

TABLE XXXIX

Absorption experiment

Serum no. 4 with leucocytes, platelets and erythrocytes from donor no. 51

Leucocyte suspension from donor no. 51 incubated with	Dilution of serum no. 4			
	1/1	1/2	1/4	1/8
serum no. 4 before absorption:	+++	++	+	-
serum no. 4 (0.5 ml) after absorption with: 1×10^8 leucocytes from donor no. 51	-	-	-	-
1.5×10^8 platelets from donor no. 51	++	+	-	-
3×10^8 platelets from donor no. 51	+	-	-	-
6×10^8 platelets from donor no. 51	-	-	-	-
3×10^8 erythrocytes from donor no. 51	+++	++	+	-

Placental tissue

In order to find out whether these antigens occurred also in placental tissue the placenta from a randomly chosen patient (Mrs.K.), in whose serum no antibodies against leucocytes were demonstrable was used. Since it is not possible to separate the maternal from the foetal part of the placenta, a serum was used which gave a positive agglutination test with the leucocytes from both Mrs.K. and her child (serum no. 2) and a serum which gave a negative reaction with both (serum no. 5). The latter was used to exclude the possibility that the absorption might be caused by a non-specific factor. Table XL shows that placental tissue is in fact able to absorb an antibody from the serum, if the corresponding antigen is present on the leucocytes of the mother and the child. Since the reaction of serum no. 5, after absorption with the placental tissue, remained positive with the leucocytes of donor N.,

one may conclude that the reaction is specific. This experiment was repeated 5 times with placentae from other women and with other sera, whereby the same pattern of results was obtained. It thus appears that the antigens against which the leucocyte antibodies are directed are present not only on the leucocytes and platelets, but also in the placental tissue.

TABLE XL

Demonstration of the presence of leucocyte antigens in placental tissue

	Agglutination reaction of serum no. 2 with the leucocytes of		
	mother K.	child K.	donor N.
Before absorption	+++	+++	++
After absorption with placenta	-	-	-

	Agglutination reaction of serum no. 5 with the leucocytes of		
	mother K.	child K.	donor N.
Before absorption	-	-	++
After absorption with placenta	-	-	++

Kidney tissue

Table XLI shows that the agglutinins of the sera nos. 1, 12 and 18, which were able to agglutinate the leucocytes of patient J. K., were absorbed specifically out of the serum by a homogenate of kidney tissue of patient J. K. This experiment was repeated 4 times using other serum-kidney combinations with identical results.

TABLE XLI

Demonstration of the presence of leucocyte antigens in kidney tissue

	Agglutination reaction of serum no:					
	1	12	18	4	26	34
with leucocytes from patient J. K.	+++	+++	+++	-	-	-
leucocytes from donor no. 79	+++	++	++	++	+	++
The sera were retested after absorption with kidney tissue of patient J. K. with						
leucocytes from patient J. K.	-	-	-	-	-	-
leucocytes from donor no. 79	-	-	-	++	++	++

Discussion and conclusions

In this section evidence has been presented that the leucocyte group substance can be found in cells and tissues quite different from the leucocyte.

The observation that the agglutination pattern of granulocytes and lymphocytes can vary from one serum-leucocyte combination to another can be explained in different ways. Putting aside the possibility that the pattern was produced by an artefact, which is improbable because the results were reproducible, the differences observed could be explained by:

1) the existence of agglutinins of anti-granulocyte and anti-lymphocyte specificity, as has been suggested by André et al. (1);
2) the existence of differences between individuals as to the amount of antigen present on granulocytes and lymphocytes; or
3) a combination of these two possibilities.

It is also apparent that the number of thrombocytes present in the cell suspension has no clear-cut influence on the outcome of the agglutination reaction. This makes it unlikely that the different results obtained with Dausset's and van Loghem's tests are due to the absence or presence of platelets respectively.

Up till now erythrocytes are the only cell type on which leucocyte group substance has not been demonstrated, either by direct or by indirect means. One wonders if this would also apply to the red-cell precursors or whether these actually do possess leucocyte group substance, but lose it during their maturation. However, only a small number of cell species has so far been investigated and the possibility that other tissues also lack the leucocyte group substance is a real one.

The presence of leucocyte group substance on platelets has been conclusively shown by the absorption experiments. This observation is in good agreement with the agglutination experiments of Dausset (23). He could demonstrate an almost perfect correlation between the results of the leucocyte agglutination test (using leucocytes from defibrinated blood) and a thrombocyte agglutination technique when the leucocytes and the thrombocytes were obtained from the same donor. Bosch et al. could prove the same point by survival time studies (5). They showed that the survival time of donor thrombocytes transfused into a patient was shortened when the leucocytes of the donor were agglutinated by the serum of the recipient. This does not imply that all leucocyte iso-antigens are present on the thrombocyte and vice versa. Moulinier (53) and van Loghem et al. (45) demonstrated the presence of group-specific antigens on the thrombocyte which were not present on the leucocyte.

The antigens also appear to be present in placental tissue. It seems unlikely that the results of our experiments are due to leucocytes or fragments of leucocytes present in the placenta. In the first place few leucocytes were seen in the histological preparations of the placentae which were used for the absorption experiments. Secondly, the homogenised placental tissue contained per gram of wet weight roughly the same amount of leucocyte group substance as an almost pure leucocyte suspension. This would imply that the homogenised placental tissue consisted mainly of leucocytes, which in view of the above-mentioned histological findings seems hardly plausible. Theoretically the

leucocyte agglutinins could be formed because the mother is immunised by cells of the foetal part of the placenta, which enter the blood stream of the mother during pregnancy. As no leucocyte agglutinins were found in a group of 154 primiparae this seems rather unlikely. In a beautiful, carefully controlled study Borst-Eilers could demonstrate that shortly after delivery the number of foetal cells in the maternal blood stream rises significantly (4). It seems reasonable to assume that these red cells will be accompanied by leucocytes and platelets. Whether these cells bring about the immunisation of the mother, or whether fragments of the foetal part of the placenta are responsible will be difficult to assess.

The lack of correlation between the presence of leucocyte agglutinins and the occurrence of abortion could be explained in several ways. In the first place it is not impossible that the lack of correlation is only apparent, the significance of the presence of the agglutinins being disguised by the many other factors which can cause abortion. However, it should be kept in mind that many mothers with strong leucocyte agglutinins are delivered, at term, of a normal child with a normal placenta. This could mean that the agglutinins are as a rule not cytotoxic or that they are not able to pass the placenta.

The presence of the leucocyte group substance on skin or buccal epithelial cells has not been demonstrated by direct means, e.g. absorption experiments. However, the experiments of Bosch et al. and van Rood et al. have given fairly conclusive, though indirect evidence of the presence of leucocyte group substance in skin (5, 64). These authors performed a number of split-skin homograft transplantations and showed that after the rejection of the graft, the survival time of the platelets of the skin donor, when transfused into the skin recipient, was always shortened, although no leucocyte antibodies could be demonstrated. After this survival time experiment, in which the transfusion probably acted as a booster, antibodies against leucocytes and platelets could be demonstrated. Since the recipients of the skin were men who had not previously been immunised by blood transfusions, it seems probable that the skin transplantation was responsible for the formation of the antibodies which in their turn were responsible for the shortening of the platelet survival time. As these antibodies were able after the booster injection to agglutinate leucocytes, and as such agglutinins are in our experience never formed after only one thrombocyte transfusion, it seems logical to assume that the leucocyte group substance is present in the skin.

The demonstration of the leucocyte group substance in kidney tissue might be of importance in kidney homotransplantation. Dausset has reported that of the six kidney homotransplantations performed by Hamburger only the two which were leucocyte-compatible survived (24). On the other hand van Rood et al. were not able to achieve a significant prolongation of the survival of split-skin transplants from skin donors who were leucocyte-compatible with the recipient (64).

Chapter five

GENERAL DISCUSSION

Although the aims formulated in chapter one have been successfully realised, it seems appropriate to discuss whether the means used were the most suitable. For instance, the defects of the leucocyte agglutination test have been discussed in extenso in chapter three. After illustrating these shortcomings, chapter three ended with the remark that the test procedures were not changed in order to preserve the homogeneity of the observations; moreover because even with these techniques a sufficient number of sera giving fairly reproducible results was available.

It might be argued that it would have been more logical first to investigate more extensively the possibilities of improving the technique.

This was not done, because it seems premature to aim at the improvement of a technique if one does not know when the test ought to give a positive and when a negative result, i.e. in this case when one is unable to recognise a leucocyte group. In chapter four is described how even with this imperfect test a leucocyte group can be recognised. In this manner the vicious circle: imperfect technique, therefore no leucocyte group, therefore no improvement of the technique, could be broken.

The essential feature of the method developed in order to recognise a leucocyte group was the use of statistical methods to overcome the shortcomings of an imperfect technique. These methods were rather time-consuming, but this was unavoidable, as can be illustrated by the following: it would have been easier to use only sera which on cross-absorption appeared to recognise only one leucocyte group. However, to be able to perform a cross-absorption experiment it is necessary to have at one's disposal donors whose leucocyte agglutination pattern with the serum under study is known. This means that one is forced to test the serum with a leucocyte panel, but it is of course of little use to perform the time-consuming cross-absorption experiment with a serum which gives a high percentage of dubious results. To be able to exclude such sera it is necessary to test the sera with a relatively large number of leucocyte samples. As the use of a large panel was thus unavoidable, the help of the computers enabled us to select with a minimum of trouble the sera which might be useful for the recognition of a leucocyte group.

A second essential, though unconventional, feature in the approach to the recognition of a leucocyte group was the use of a panel of leucocytes from women who had formed agglutinins having group Four specificity and from their husbands. When the specificity of an antibody against red cells has to be determined a panel of donors of known group-specificity is normally used. As the group-specificity of the leucocytes was unknown this was not possible. The use of the leucocytes of the couples overcame this difficulty.

These two points, combined with the circumstance that sera with leucocyte agglutinins formed during pregnancy often recognise only

one leucocyte group, were the most important factors which made the recognition of a leucocyte group possible.

Although the results obtained give an adequate answer to the question formulated in chapter one, one wonders to what extent the foregoing observations are of importance for the clinician.

The importance of these antibodies for the occurrence of non-haemolytic transfusion reactions has already been mentioned. It lies outside the scope of this discussion to relate the observations which proved the causal relationship between the presence of leucocyte agglutinins in the recipient and non-haemolytic transfusion reactions (for such a review see Walford, 74). These transfusion reactions are in general not serious, although the occurrence of fibrinolysis has been reported (9).

Brittingham has, however, shown that the injection of small quantities of serum containing leucocyte agglutinins can cause serious transfusion reactions (8). Leucocyte agglutinins formed during pregnancy can remain present in the blood up to several years after delivery. For that reason dangerous reactions can occur after the (rapid) infusion of the blood of female donors who have been pregnant (62).

As the formation of these antibodies is in so many respects reminiscent of that of the Rhesus antibodies, it is logical to look for an analogy of the morbus haemolyticus neonatorum. This affliction is indeed known as the "neutropenia of the newborn". Table XLII* shows the most important data from the cases known to me. Interesting, and unexplained, is the observation that the minimal granulocyte count is reached only 3 - 22 days post partum. The most intriguing point seems to be, that a leucopenia rarely occurs, but in most instances only a neutropenia. This could be explained as follows:

1) there exists an anti-granulocyte antibody which can pass the placenta, and an anti-lymphocyte antibody which cannot; or
2) the production of lymphocytes is sufficient to compensate for the destruction whereas the production of granulocytes is not; or
3) the leucocyte group substance is present on the granulocytes at birth but develops on the lymphocytes at a later date.

The observation that leucocyte group substance is present on the platelets and that the survival time of the platelets after transfusion is shortened in the presence of leucocyte agglutinins implies that the leucocyte agglutination reaction might be of importance in platelet transfusion practice (5). However, to be really useful the test must be both more reliable besides being complemented with techniques able to recognise thrombocyte antibodies and incomplete leucocyte antibodies and accompanied by a more complete insight into the structure of leucocyte groups. As platelet transfusions have a definite though limited field of indication in the therapy of haemorrhagic diathesis (6, 18, 61) it might be well worthwhile to pursue this line of investigation.

It is evident that leucocyte groups might also be useful in other fields such as anthropology and forensic medicine.

The possible significance of leucocyte groups for transplantation immunity has already been pointed out. Although as yet no definite information on this point is available the study of leucocyte groups forms a hopeful approach to this problem.

Leucocyte group Four fulfills the criteria of a gene-marker. As

there is no close linkage with one of the other gene-markers so far studied, the addition of leucocyte group Four can be looked upon as a real acquisition in our efforts to map the chromosome. Furthermore the data presented in chapter four indicate that the recognition of other leucocyte groups will soon be possible.

Chapter six

SUMMARY

This study describes the development of a method for the recognition and definition of leucocyte group Four. The method can also be applied to the detection of other leucocyte groups. Essential factors in the method were:

a) the use of leucocyte agglutinins formed during pregnancy;
b) an insight into the shortcomings of the agglutination test;
c) the use of statistical methods to overcome these shortcomings; and
d) the use of a panel consisting of the leucocytes of the women who had formed the agglutinins, and of those of their husbands.

In chapter one the pertinent literature is reviewed, especially that concerning the role of pregnancy in the formation of leucocyte agglutinins and the arguments in favour of the existence of leucocyte groups.

In chapter two a detailed description of the materials and methods is given.

Chapter three is subdivided into three sections. The first section, after reviewing the literature, details the influence of time, temperature and number of leucocytes on the outcome of the reaction. Furthermore the two agglutination tests, i.e. with leucocytes from defibrinated and from EDTA blood, are compared with regard to the percentage of dubious results, the percentage of positive results and the reproducibility. The second section describes the experiments which showed that these agglutinins are in all probability iso-immune antibodies directed against an antigen absent from the leucocytes of the women who had formed the agglutinins but present on the leucocytes of their husbands. Some of the physico-chemical and immunological properties of the agglutinins are described. The third section contains the data concerning the correlation between the number of cases in which leucocyte agglutinins were demonstrable and the number of previous pregnancies, the influence of previous blood transfusions, and the techniques used. No correlation was found between the presence of leucocyte agglutinins and a history of abortion.

Chapter four is divided into two sections. The first section describes the methods used to select, from a group of 66 sera, those which might recognise a leucocyte group. These methods included the use of Fisher's 2 x 2 test and of electronic computers. Twelve sera selected in this way were studied and it was shown that they recognised a leucocyte group, designated as leucocyte group Four because the existence of three other leucocyte groups had been postulated before. However, it is not impossible that leucocyte group Four is identical with the leucocyte group Mac described by Dausset (23). Leucocyte group Four is determined by the antigens 4^a and 4^b which behave as if they are determined by a single pair of alleles. Both can be recognised expressed in the heterozygote but there is evidence of an antigen dosage effect. In the second section the leucocyte group substance

is demonstrated to be present not only on leucocytes but also on platelets, and in placenta and kidney tissue.

In chapter five the extent is discussed to which the procedure followed was rational. A review is also given of the possible clinical significance of the formation of leucocyte agglutinins during pregnancy in general, and the possible usefulness of the recognition of leucocyte group Four in particular.

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TABLES
AND
FIGURES

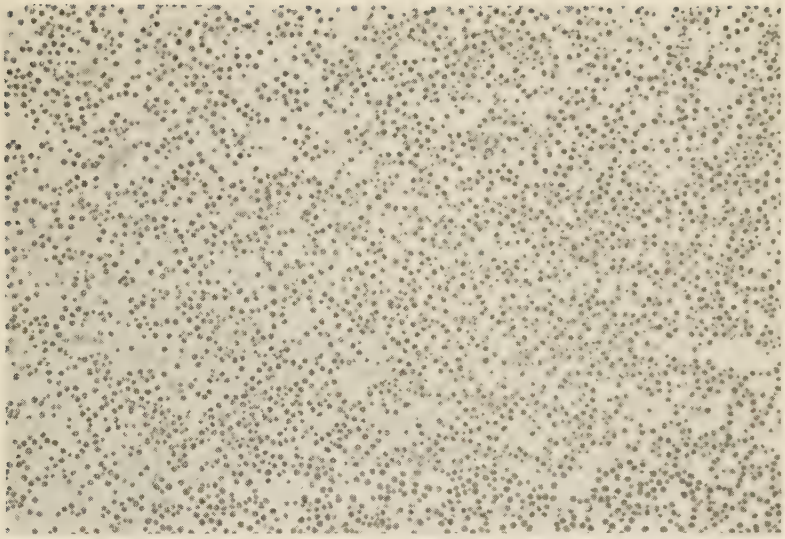

Figure 2a
Leucocyte agglutination test (80 x). Dausset's method.
Negative reaction

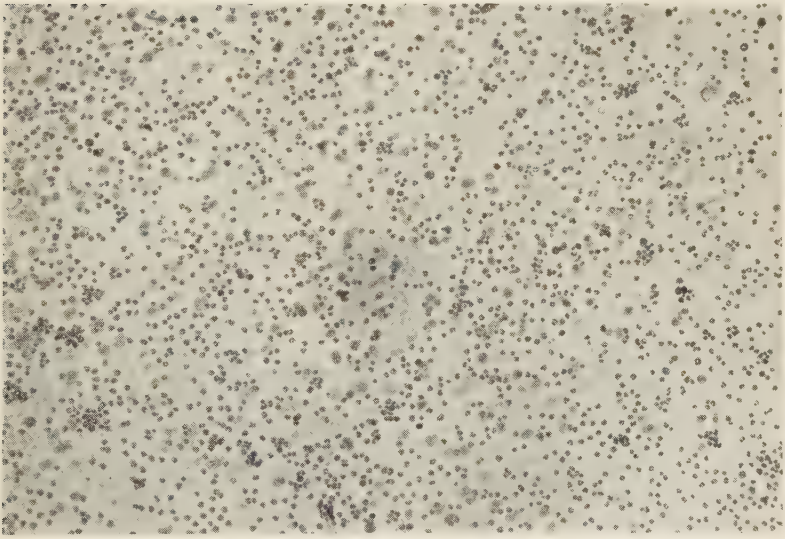

Figure 2b
Leucocyte agglutination test (80 x). Dausset's method.
Weakly positive reaction

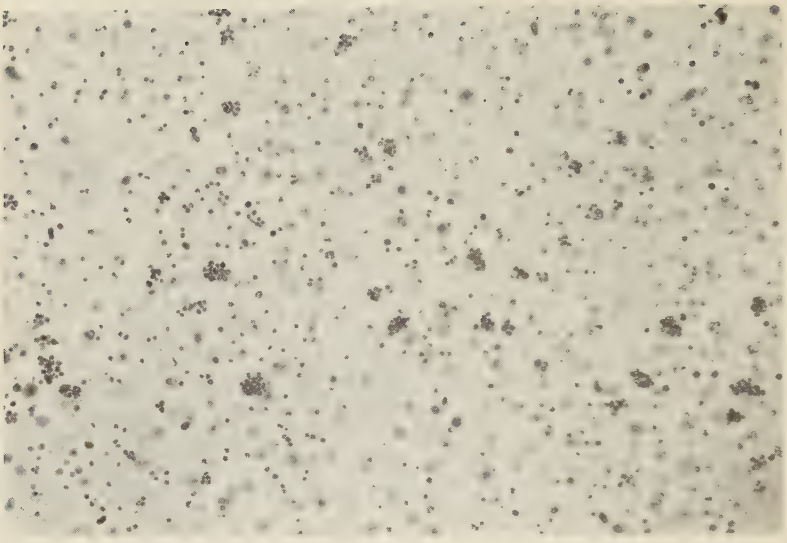

Figure 2c
Leucocyte agglutination test (80 x). Dausset's method.
+ positive reaction

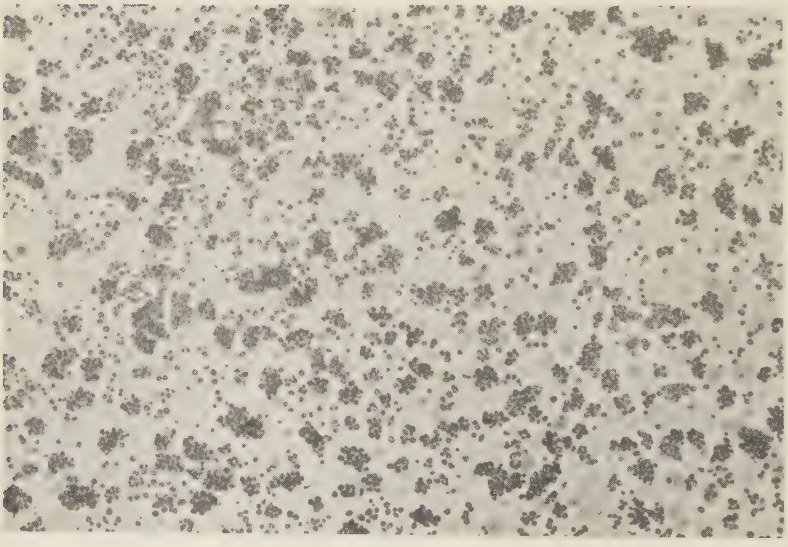

Figure 2d
Leucocyte agglutination test (80 x). Dausset's method.
++ positive reaction

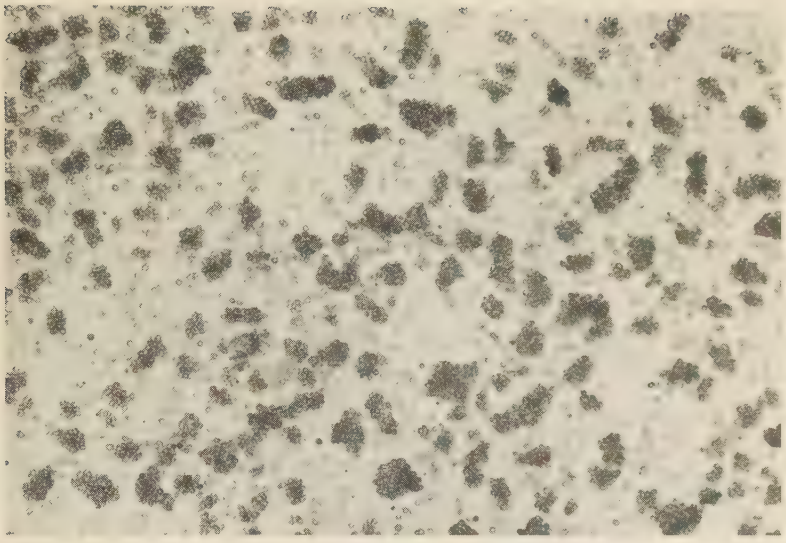

Figure 2e
Leucocyte agglutination test (80 x). Dausset's method.
+++ positive reaction

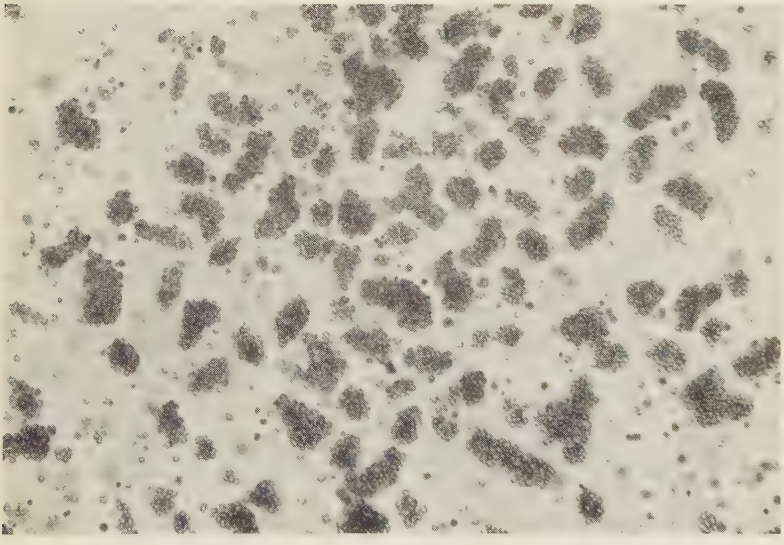

Figure 2f
Leucocyte agglutination test (80 x). Dausset's method.
++++ positive reaction

Figure 3a
Leucocyte agglutination test (80 x). van Loghem's method.
Negative reaction

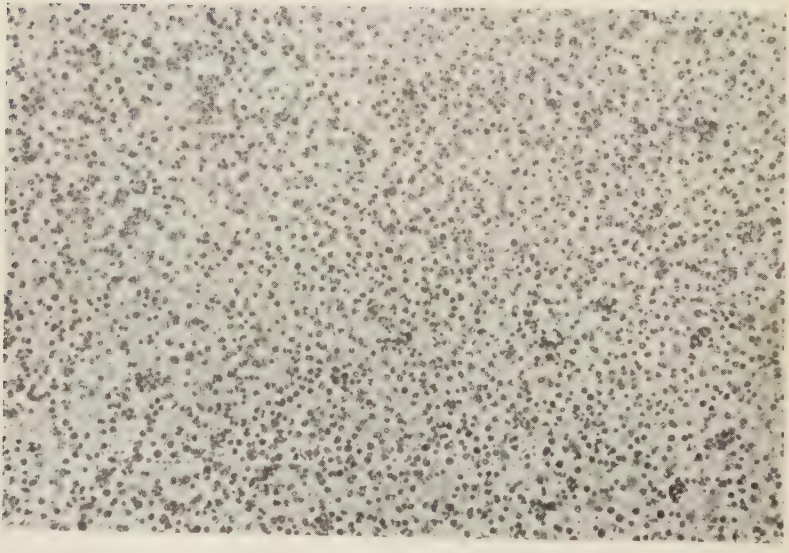

Figure 3b
Leucocyte agglutination test (80 x). van Loghem's method.
Weakly positive reaction

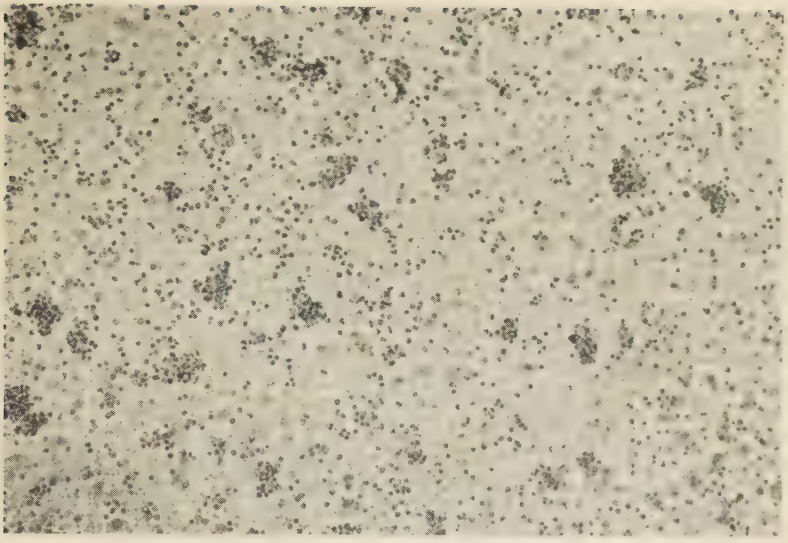

Figure 3c
Leucocyte agglutination test (80 x). van Loghem's method.
+ positive reaction

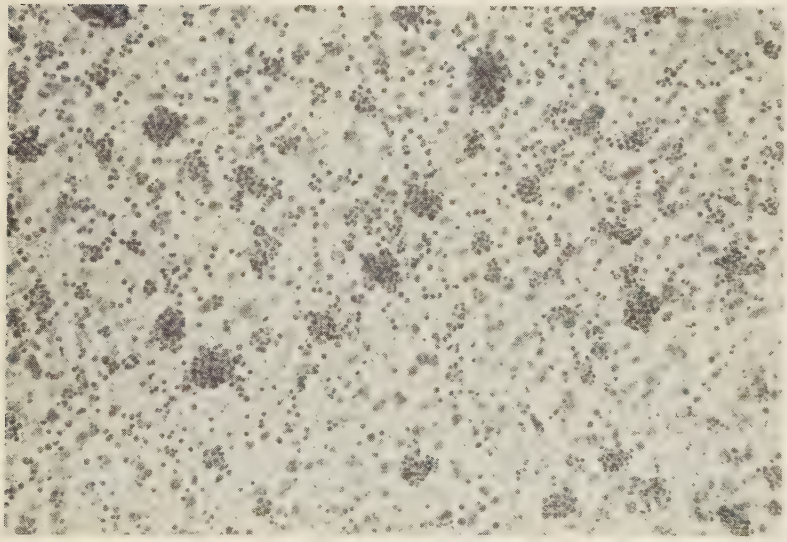

Figure 3d
Leucocyte agglutination test (80 x). van Loghem's method.
++ positive reaction

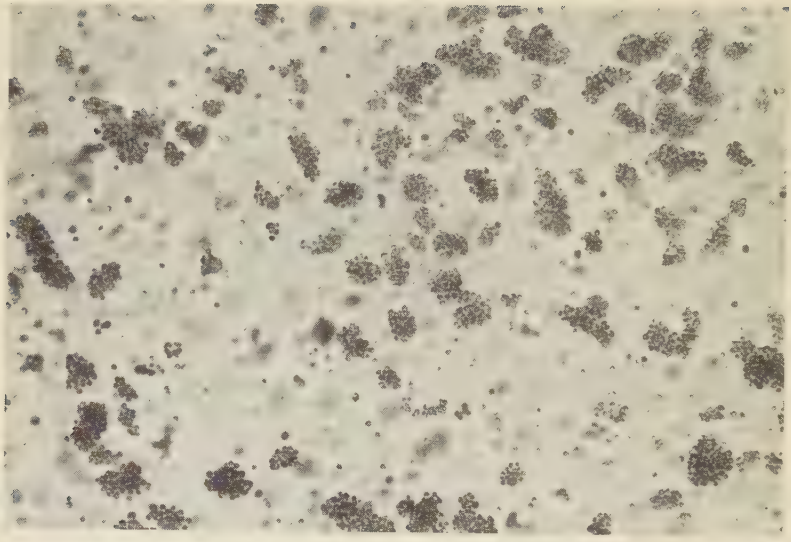

Figure 3e
Leucocyte agglutination test (80 x). van Loghem's method.
+++ positive reaction

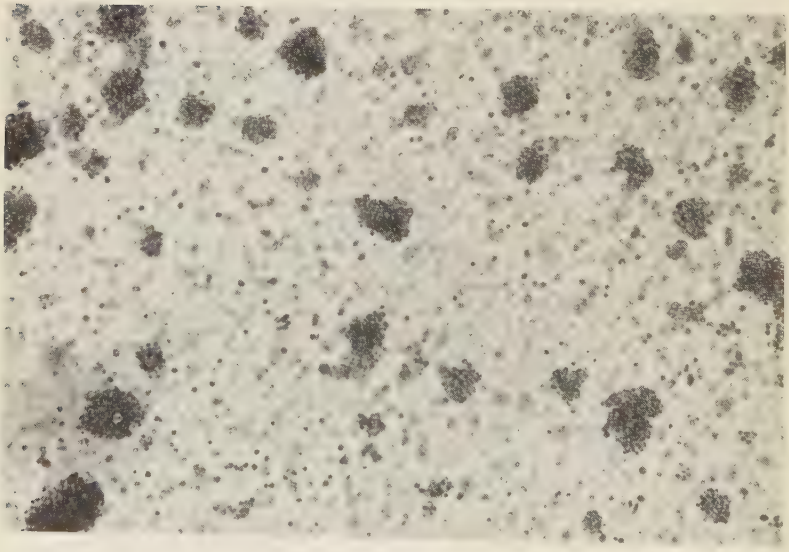

Figure 3f
Leucocyte agglutination test (80 x). van Loghem's method.
++++ positive reaction

TABLE XXXVIII

Agglutination pattern of isolated granulocytes and lymphocytes

	Dilution of serum no. 4											
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	score	leucocytes per mm^3	number of platelets of suspension
Donor no. 98 Normal leucocyte suspension	+++	++	+	-	-	-	-	-	-	9	9000	420, 000
Granulocyte suspension platelet-poor	+++	++	+	-	-	-	-	-	-	9	8700	30, 000
Granulocyte suspension platelet-rich	+++	++	+	-	-	-	-	-	-	9	8000	330, 000
Lymphocyte suspension platelet-poor	+++	++	+	-	-	-	-	-	-	9	8200	90, 000
Lymphocyte suspension platelet-rich	+++	++	+	-	-	-	-	-	-	9	8200	540, 000
	Dilution of serum no. 4											
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256			
Donor no. 51 Normal leucocyte suspension	+++	+++	++	+	-	-	-	-	-	13	9600	420, 000
Granulocyte suspension platelet-poor	+++	++	+	+	-	-	-	-	-	11	9400	50, 000
Granulocyte suspension platelet-rich	+++	++	+	+	-	-	-	-	-	11	8000	420, 000
Lymphocyte suspension platelet-poor	-	-	-	-	-	-	-	-	-	0	9400	60, 000
Lymphocyte suspension platelet-rich	-	-	-	-	-	-	-	-	-	0	7500	340, 000
	Dilution of serum no. 1											
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256			
Donor no. 101 Normal leucocyte suspension	+++	++	+	+	(+)	-	-	-	-	12	8100	520, 000
Granulocyte suspension platelet-poor	+	(+)	-	-	-	-	-	-	-	3	8900	40, 000
Granulocyte suspension platelet-rich	+	(+)	-	-	-	-	-	-	-	3	8800	480, 000
Lymphocyte suspension platelet-poor	++	+++	+++	++	++	++	+	+	-	24	8600	160, 000
Lymphocyte suspension platelet-rich	++	+++	++	++	++	+	+	+	-	22	8100	520, 000

APPENDIX

Distribution of leucocyte group Four and the blood groups ABO, MNS, P, Rhesus, Kell, Duffy and Kidd in the members of the families shown in table XXXV

				Anti-4^a				Anti-4^b												
				Serum no:																
			sex	22	34	35	36	12	15	18	23	48								
fam. 1	I	1	m	0	0	0	0	4	3	4	3	4	4^b4^b	A_2	MN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a-	
		2	f	0	0	0	0	4	3	4	4	4	4^b4^b	A_1	MN		P_1+	$C\bar{c}D\bar{e}\bar{e}$	K−	Fy^a-	
	II	1	m	0	0	0	0	4	3	3	3	3	4^b4^b	A_1	MM		P_1+	$C\bar{c}DE$	K−	Fy^a-	
		2	m	0	0	0	0	4	2	3	3	3	4^b4^b	A_1	MN		P_1+	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a-	
		3	f	0	0	0	0	4	4	5	4	4	4^b4^b	O	MN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a-	
		4	m	0	0	0	0	4	3	3	3	2	4^b4^b	O	NN		P_1+	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a-	
		5	m	0	0	0	0	4	2	3	3	3	4^b4^b	A_2	MM		P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a-	
		6	f	0	0	0	0	4	2	4	3	3	4^b4^b	A_2	MM		P_1+	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a-	
		7	f	0	0	0	0	4	3	3	3	3	4^b4^b	A_2	NN		P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a-	
fam. 2	I	1	m	3	2	1	4	4	3	4	3	3	4^a4^b	A_1	MM	S+	P_1-	$C\bar{c}DE$	K−	Fy^a+	
		2	f	0	0	0	0	4	1	4	3	3	4^b4^b	O	MM	S+	P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a-	
	II	1	m	2	2	0	1	4	1	4	4	4	4^a4^b	A_1	MM	S+	P_1-	$\bar{c}\bar{c}DE$	K−	Fy^a+	
		2	f	3	1	0	3	4	3	4	4	4	4^a4^b	B	MM	S−	P_1+	$C\bar{c}DE$	K−	Fy^a-	
		3	f	0	0	0	0	4	2	4	4	3	4^b4^b	O	MM	S+	P_1+	$C\bar{c}DE$	K−	Fy^a+	(see fig.7*)
		4	m	0	0	0	0	4	2	4	4	4	4^b4^b	O	MM	S+	P_1-	$C\bar{c}D\bar{e}$	K−	Fy^a+	
	III	1	f	2	2	0	2	4	3	4	4	4	4^a4^b	A_1	MM	S+	P_1-	$\bar{c}\bar{c}DE$	K−	Fy^a-	
		2	m	0	0	0	0	4	3	4	4	4	4^b4^b	A_1B	MM	S+	P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		3	f	3	4	0	4	4	3	4	4	4	4^a4^b	O	MM	S+	P_1-	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		4	f	0	0	0	0	4	3	4	4	4	4^b4^b	O	MM	S+	P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a-	
fam. 3	I	1	m	4	4	4	3	0	0	0	0	0	4^a4^a	O	MM		P_1+	$C\bar{c}DE$	K−	Fy^a+	
		2	f	4	1	0	3	3	2	3	2	1	4^a4^b	A_2	MM		P_1-	$C\bar{c}D\bar{e}$	K−	Fy^a+	
	II	1	f	4	3	1	3	0	0	0	0	0	4^a4^a	O	MM		P_1-	$C\bar{c}DE$	K−	Fy^a+	
		2	f	3	2	0	4	3	2	3	0	1	4^a4^b	A_2	MM		P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		3	m	4	3	3	3	3	2	3	2	3	4^a4^b	O	MM		P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		4	m	4	4	0	3	0	0	0	0	0	4^a4^a	A_2	MM		P_1+	$C\bar{c}DE$	K−	Fy^a+	
fam. 4	I	1	m	0	0	0	0	4	3	4	4	4	4^b4^b	O	MN		P_1+	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		2	f	5	4	4	3	0	0	0	0	0	4^a4^a	O	NN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a+	
	II	1	f	4	3	2	3	3	3	4	3	2	4^a4^b	O	NN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a+	
		2	f	4	3	2	3	3	1	3	3	1	4^a4^b	O	MN		P_1-	$C\bar{c}D\bar{e}$	K−	Fy^a+	
		3	m	3	3	1	3	3	2	3	3	2	4^a4^b	O	MN		P_1-	$\bar{c}\bar{c}DE$	K−	Fy^a+	
		4	m	5	4	3	3	4	3	3	3	4	4^a4^b	O	MN		P_1+	$C\bar{c}DE$	K−	Fy^a+	
fam. 5	I	1	m	4	4	3	3	0	0	0	0	0	4^a4^a	A_1	MM		P_1+	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a+	
		2	f	4	3	1	0	3	2	2	2	2	4^a4^b	A_1	MN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a+	
	II	1	m	5	3	2	3	4	3	4	4	4	4^a4^b	O	MN		P_1+	$\bar{c}\bar{c}DE$	K−	Fy^a+	
		2	m	4	3	2	3	3	3	3	3	3	4^a4^b	A_1	MM		P_1-	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a+	
		3	f	4	3	3	2	4	3	3	3	1	4^a4^b	A_1	MN		P_1+	$\bar{c}\bar{c}d\bar{e}$	K−	Fy^a+	
		4	f	3	2	0	2	2	0	3	3	2	4^a4^b	O	MM		P_1+	$\bar{c}\bar{c}DE\bar{e}$	K−	Fy^a+	

				Anti-4^a				Anti-4^b										
				Serum no:														
			sex	22	34	35	36	12	15	18	23	48						
fam. 6	I	1	m	3	1	2	3	4	3	3	4	3	4^a4^b	O	MN	P_1+	C$\bar{c}$D$\bar{e}$	K–	Fy^a–
		2	f	0	0	0	0	4	2	3	3	3	4^b4^b	A_1	MN	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
	II	1	m	0	0	0	0	4	2	3	3	3	4^b4^b	A_2	NN	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a–
		2	m	3	1	1	3	3	3	3	3	2	4^a4^b	A_2	MN	P_1+	C$\bar{c}$D$\bar{e}$	K–	Fy^a+
		3	f	0	0	0	0	3	2	3	4	3	4^b4^b	A_2	MM	P_1+	C$\bar{c}$D$\bar{e}$	K–	Fy^a–
		4	f	0	0	0	0	3	2	3	3	3	4^b4^b	A_1	MM	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		5	m	0	0	0	0	4	3	4	3	4	4^b4^b	A_1	NN	P_1+	C$\bar{c}$D$\bar{e}$	K–	Fy^a+
fam. 7	I	1	m	0	0	0	0	4	3	4	3	3	4^b4^b	A_1	MN	P_1+	$\bar{c}\bar{c}$DE	K+	Fy^a+
		2	f	0	0	0	0	3	1	3	3	2	4^b4^b	A_1	MM	P_1–	C$\bar{c}$D$\bar{e}$	K–	Fy^a+
	II	1	m	0	0	0	0	3	2	3	3	2	4^b4^b	A_1	MN	P_1+	$\bar{c}\bar{c}$DE	K+	Fy^a+
		2	f	0	0	0	0	3	1	3	3	1	4^b4^b	A_1	MM	P_1+	C$\bar{c}$DE	K+	Fy^a+
		3	f	0	0	0	0	3	2	3	3	3	4^b4^b	A_1	MN	P_1+	C$\bar{c}$DE	K–	Fy^a+
		4	f	0	0	0	0	4	2	3	3	3	4^b4^b	A_1	MM	P_1+	C$\bar{c}$DE	K–	Fy^a+
		5	f	0	0	0	0	4	2	3	4	3	4^b4^b	A_1	MN	P_1+	C$\bar{c}$DE	K+	Fy^a+
		6	m	0	0	0	0	3	2	2	2	1	4^b4^b	O	MN	P_1+	C$\bar{c}$DE	K–	Fy^a+
fam. 8	I	1	m	4	4	2	2	3	2	3	3	3	4^a4^b	A_1	MN S+	P_1–	$\bar{c}\bar{c}$DE	K–	Fy^a+
		2	f	4	3	2	3	3	2	3	3	3	4^a4^b	O	NN S–	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
	II	1	m	4	3	2	3	3	2	3	3	2	4^a4^b	A_1	MN S+	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		2	f	5	4	3	3	0	0	0	0	0	4^a4^a	A_1	MN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		3	f	5	4	3	3	0	0	0	0	0	4^a4^a	A_1	NN S–	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		4	m	5	4	3	3	0	0	0	0	0	4^a4^a	O	MN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		5	f	4	3	2	3	4	3	4	3	3	4^a4^b	O	MN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		6	m	4	4	3	3	0	0	0	0	0	4^a4^a	A_1	MN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		7	m	4	3	1	3	3	0	3	3	3	4^a4^b	A_1	MN S+	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		8	f	0	0	0	0	4	2	3	3	3	4^b4^b	O	NN S–	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a–
		9	m	0	0	0	0	4	2	4	4	3	4^b4^b	A_1	NN S–	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
fam. 9	I	1	m	0	0	0	0	4	1	4	3	2	4^b4^b	B	MM S–	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		2	f	5	5	3	4	0	0	0	0	0	4^a4^a	O	MM S+	P_1+	C$\bar{c}\bar{d}\bar{e}$	K–	Fy^a–
	II	1	m	3	3	1	2	3	1	4	2	2	4^a4^b	O	MM S+	P_1+	C$\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		2	m	4	4	3	4	4	2	3	3	3	4^a4^b	B	MM S+	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		3	f	5	5	4	4	4	5	5	5	4	4^a4^b	O	MM S+	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
fam. 10	I	1	m	4	4	3	2	4	3	4	4	3	4^a4^b	A_1	MN S–	P_1+	CCD$\bar{e}$	K–	Fy^a+
		2	f	4	5	4	4	0	0	0	0	0	4^a4^a	O	MN S+	P_1+	C$\bar{c}$DE	K–	Fy^a+
	II	1	m	5	3	3	2	4	3	3	3	3	4^a4^b	A_1	NN S–	P_1+	C$\bar{c}$DE	K–	Fy^a+
		2	f	4	4	3	1	4	2	4	4	2	4^a4^b	A_1	MN S+	P_1+	C$\bar{c}$DE	K–	Fy^a+
fam. 11	I	1	m	2	2	0	0	3	3	3	3	1	4^a4^b	A_1	MN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		2	f	4	3	2	4	3	2	3	3	3	4^a4^b	O	NN S–	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
	II	1	f	4	3	1	3	4	2	3	2	3	4^a4^b	A_1	NN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a–
		2	f	4	3	1	3	3	1	3	1	3	4^a4^b	A_1	NN S+	P_1+	$\bar{c}\bar{c}\bar{d}\bar{e}$	K–	Fy^a+
		3	f	2	3	2	3	3	0	3	3	2	4^a4^b	O	NN S+	P_1–	$\bar{c}\bar{c}$DE	K–	Fy^a+
		4	m	4	3	2	3	3	1	3	3	3	4^a4^b	A_1	MN S–	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a+
		5	f	4	2	0	1	5	3	4	3	4	4^a4^b	O	NN S+	P_1+	$\bar{c}\bar{c}$DE	K–	Fy^a–

				Anti-4^a				Anti-4^b											
				Serum no:															
			sex	22	34	35	36	12	15	18	23	48							
fam. 12	I	1	m	3	2	1	3	3	1	2	3	3	4^a4^b	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	f	0	0	0	0	4	2	4	3	4	4^b4^b	A_2	MM S+	P_1+	CCD$\bar{e}$	K−	Fy^a+	
	II	1	m	0	0	0	0	4	2	4	4	2	4^b4^b	A_1	MM S+	P_1+	CCD$\bar{e}$	K−	Fy^a+	
		2	f	4	3	1	3	3	2	3	2	2	4^a4^b	O	MM S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		3	f	0	0	0	0	4	3	3	1	2	4^b4^b	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		4	m	4	2	1	2	2	2	3	2	1	4^a4^b	A_1	MN S+	P_1−	$\bar{c}\bar{c}$DE	K−	Fy^a+	
		5	m	4	3	3	3	4	0	3	3	1	4^a4^b	A_2	MM S+	P_1−	$\bar{c}\bar{c}$DE	K−	Fy^a+	(see fig.8*)
		6	f	0	0	0	0	4	2	3	3	3	4^b4^b	A_1	MM S−	P_1+	CCD$\bar{e}$	K−	Fy^a+	
	III	1	m	5	3	3	3	4	2	4	4	4	4^a4^b	A_2	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	m	5	3	3	3	4	3	3	4	4	4^a4^b	A_2	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		3	f	3	3	2	3	3	1	2	2	2	4^a4^b	A_2	MM S+	P_1+	CCD$\bar{e}$	K−	Fy^a+	
		4	f	0	0	0	0	3	1	4	5	3	4^b4^b	A_1	MM S+	P_1+	CCD$\bar{e}$	K−	Fy^a+	
		5	f	0	0	0	0	4	3	4	4	4	4^b4^b	O	MM S+	P_1+	$\bar{c}\bar{c}$DE	K−	Fy^a+	
		6	m	0	0	0	0	4	2	4	3	3	4^b4^b	A_1	MN S+	P_1+	$\bar{c}\bar{c}$DE	K−	Fy^a+	
fam. 13	I	1	m	3	3	1	2	0	0	0	0	1	4^a4^a	A_1	MN S−	P_1+	CCD$\bar{e}$	K−	Fy^a−	
		2	f	0	0	0	0	4	3	4	4	4	4^b4^b	O	MN S−	P_1+	$\bar{c}\bar{c}$D$\bar{e}$	K−	Fy^a+	
	II	1	m	4	3	0	2	4	1	4	3	2	4^a4^b	A_1	MN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
		2	f	3	3	2	2	4	3	4	4	4	4^a4^b	O	NN S−	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
		3	f	3	1	1	0	4	2	4	4	3	4^a4^b	O	MN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
		4	f	2	2	1	0	4	3	4	4	4	4^a4^b	A_1	MN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		5	f	2	1	1	0	5	3	4	4	3	4^a4^b	O	NN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		6	f	4	3	0	1	4	3	4	4	3	4^a4^b	O	MN S−	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
fam. 14	I	1	m	3	2	0	3	3	1	2	1	1	4^a4^b	A_1	MM S+	P_1+	$\bar{c}\bar{c}$D$\bar{e}$	K+	Fy^a+	
		2	f	5	4	3	3	0	0	0	0	0	4^a4^a	A_2	MM S+	P_1+	CCD$\bar{e}$	K−	Fy^a−	
	II	1	m	5	3	3	3	3	3	3	3	3	4^a4^b	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	f	5	4	3	3	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K+	Fy^a+	
		3	f	4	4	3	3	2	1	2	3	1	4^a4^b	A_1	MM S+	P_1−	C$\bar{c}$D$\bar{e}$	K+	Fy^a+	
		4	m	5	4	3	3	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K+	Fy^a−	
		5	m	5	3	3	3	3	2	3	3	2	4^a4^b	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		6	m	5	4	4	4	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1+	C$\bar{c}$D$\bar{e}$	K+	Fy^a+	
fam. 15	I	1	m	0	0	0	0	4	3	4	4	4	4^b4^b	O	MM S−	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	f	5	4	3	4	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
	II	1	m	4	3	1	3	3	1	3	1	2	4^a4^b	A_1	MM S+	P_1−	$\bar{c}\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	f	3	2	0	3	3	1	2	2	1	4^a4^b	A_1	MM S+	P_1−	CCD$\bar{e}$	K−	Fy^a−	
		3	m	4	3	0	3	3	1	3	3	2	4^a4^b	A_2	MM S+	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
		4	f	3	3	0	3	3	0	2	0	0	4^a4^b	A_2	MM S+	P_1−	$\bar{c}\bar{c}$D$\bar{e}$	K−	Fy^a+	
		5	f	4	3	2	2	3	1	3	3	2	4^a4^b	A_2	MM S+	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
		6	m	4	4	3	3	3	2	3	3	3	4^a4^b	A_2	MM S+	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
fam. 16	I	1	m	4	4	3	3	3	3	3	3	3	4^a4^b	B	NN S+	P_1+	CCD$\bar{e}$	K−	Fy^a−	
		2	f	4	3	0	3	4	3	3	3	2	4^a4^b	O	NN S+	P_1+	$\bar{c}\bar{c}$D$\bar{e}$	K−	Fy^a+	
	II	1	m	3	4	3	3	4	2	3	4	3	4^a4^b	O	NN S+	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		2	f	2	1	0	2	4	3	3	3	3	4^a4^b	A_1	NN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		3	m	5	4	5	4	0	0	0	0	1	4^a4^a	B	NN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	(see fig. 9*)
		4	f	3	1	0	1	3	0	3	2	3	4^a4^b	B	NN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a+	
		5	f	5	3	2	2	2	0	2	2	3	4^a4^b	B	NN S−	P_1+	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	
	III	1	m	4	3	2	4	3	3	4	3	3	4^a4^b	A_1	NN S−	P_1+	$\bar{c}\bar{c}$D$\bar{e}$	K−	Fy^a−	
		2	f	0	0	0	0	5	3	2	4	3	4^b4^b	O	NN S+	P_1−	C$\bar{c}$D$\bar{e}$	K−	Fy^a−	

				Anti-4^a				Anti-4^b											
			Serum no:																
			sex	22	34	35	36	12	15	18	23	48							
fam. 17	I	1	m	3	2	2	3	4	3	3	4	2	4^a4^b	O	MN S–	P_1+	C$\bar{c}$D$\bar{e}\bar{e}$	K–	Fy^a–	
		2	f	0	0	0	0	4	2	4	3	2	4^b4^b	A_1	MN S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
	II	1	m	3	2	1	3	2	2	2	2	1	4^a4^b	A_2	MN S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		2	f	0	0	0	0	3	2	3	2	2	4^b4^b	O	NN S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K+	Fy^a+	
		3	m	3	2	1	3	4	2	1	2	2	4^a4^b	A_2	MM S–	P_1+	C$\bar{c}$D$\bar{e}\bar{e}$	K–	Fy^a–	
		4	f	5	4	3	4	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1+	C$\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		5	f	3	2	2	2	2	2	2	1	1	4^a4^b	A_1	MN S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		6	m	3	3	0	4	4	3	3	3	2	4^a4^b	O	MN S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		7	f	0	0	0	0	4	3	4	4	2	4^b4^b	A_1	MN S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		8	m	0	0	0	0	4	3	4	3	2	4^b4^b	B	MN S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		9	f	4	3	3	4	4	3	2	2	2	4^a4^b	A_2	MM S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	(see fig.10*)
	III	1	m	0	0	0	0	4	3	4	4	3	4^b4^b	A_2	NN S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K+	Fy^a–	
		2	m	3	3	3	3	4	3	4	3	2	4^a4^b	O	NN S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K+	Fy^a–	
		3	f	5	3	2	3	3	2	2	1	0	4^a4^b	A_2	MM S+	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		4	f	4	3	2	4	0	0	0	0	0	4^a4^a	A_1	MM S+	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		5	m	4	4	3	3	4	3	4	4	3	4^a4^b	A_1	MM S+	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a–	
		6	f	0	0	0	0	3	2	3	3	3	4^b4^b	A_1	MM S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		7	f	4	4	3	4	4	4	3	4	4	4^a4^b	O	MN S–	P_1+	$\bar{c}\bar{c}d\bar{e}\bar{e}$	K–	Fy^a–	
		8	f	3	1	1	3	4	3	4	4	4	4^a4^b	O	MM S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		9	f	3	2	1	3	1	3	1	2	1	4^a4^b	AB	MN S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		10	f	4	3	3	4	4	4	4	3	4	4^a4^b	B	MM S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		11	m	0	0	0	0	5	3	3	4	3	4^b4^b	O	MN S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
fam. 18	I	1	m	4	3	1	1	3	2	3	3	3	4^a4^b	A_1	MN S–	P_1+	CCDE$\bar{e}$	K–	Fy^a+	
		2	f	4	2	1	0	3	0	2	2	1	4^a4^b	A_1	MM S+	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a+	
	II	1	m	4	3	3	2	4	3	3	3	3	4^a4^b	A_1	MM S–	P_1+	CCDE$\bar{e}$	K–	Fy^a+	
		2	f	0	0	0	0	4	2	3	2	3	4^b4^b	A_1	MM S–	P_1+	CCDE$\bar{e}$	K–	Fy^a+	
		3	f	4	4	3	1	3	3	3	2	2	4^a4^b	A_1	MN S+	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a+	
		4	f	0	0	0	0	4	2	3	2	3	4^b4^b	A_1	MN S–	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a–	
		5	m	3	3	2	2	3	1	3	2	1	4^a4^b	A_1	MN S–	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a+	
		6	m	0	0	0	0	4	3	4	3	4	4^b4^b	A_1	MM S+	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a–	
		7	m	4	4	2	2	2	2	3	2	3	4^a4^b	A_1	MN S–	P_1–	CCDE$\bar{e}$	K–	Fy^a+	
		8	f	4	4	3	3	0	0	0	0	0	4^a4^a	A_1	MN S–	P_1+	CCD$\bar{e}\bar{e}$	K–	Fy^a+	
		9	m	0	0	0	0	3	0	2	2	0	4^b4^b	A_1	MM S+	P_1–	CCDE$\bar{e}$	K–	Fy^a–	
		10	f	3	3	1	2	3	2	4	3	2	4^a4^b	A_1	MN S–	P_1+	CCDE$\bar{e}$	K–	Fy^a–	
fam. 19	I	1	m	0	0	0	0	4	2	4	3	3	4^b4^b	A_2	NN S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K+	Fy^a–	
		2	f	0	0	0	0	5	3	4	4	3	4^b4^b	O	MN S–	P_1–	C$\bar{c}$D$\bar{e}\bar{e}$	K–	Fy^a+	
	II	1	m	0	0	0	0	3	1	3	3	2	4^b4^b	O	MN S–	P_1+	C$\bar{c}$D$\bar{e}\bar{e}$	K–	Fy^a+	
		2	f	0	0	0	0	4	3	3	3	3	4^b4^b	O	NN S+	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a–	
		3	f	0	0	0	0	4	1	3	3	3	4^b4^b	A_2	MN S–	P_1+	$\bar{c}\bar{c}$DE$\bar{e}$	K–	Fy^a+	
		4	f	0	0	0	0	4	2	4	3	4	4^b4^b	O	NN S+	P_1–	C$\bar{c}$D$\bar{e}\bar{e}$	K+	Fy^a+	

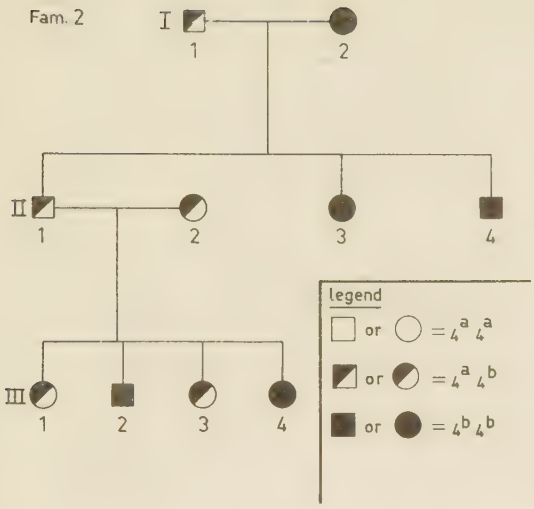

Figure 7

Pedigree of family 2

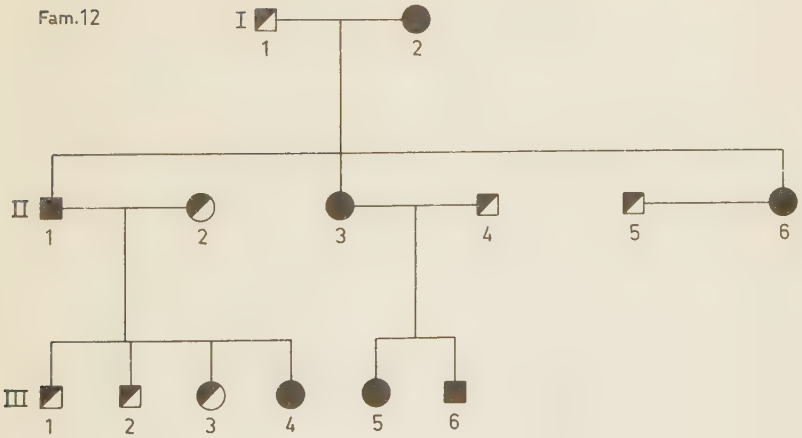

Figure 8

Pedigree of family 12

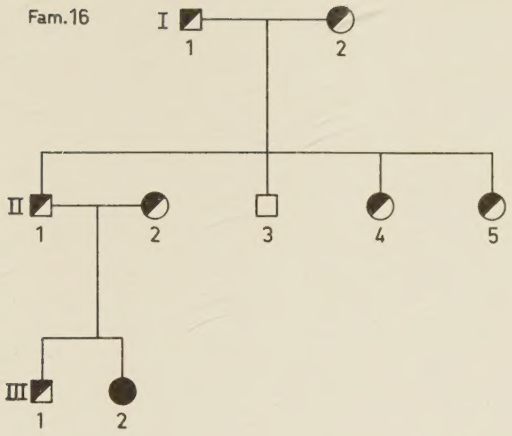

Figure 9

Pedigree of family 16

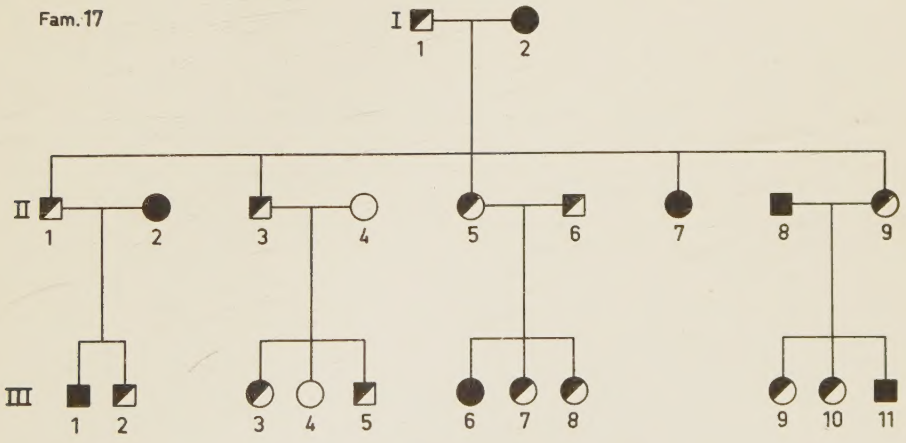

Figure 10

Pedigree of family 17